GENETIC ASSESSMENT OF TAXONOMIC UNCERTAINTY AND CONSERVATION UNITS IN PAINTED TURTLES, WITH A FOCUS ON THE AT-RISK *CHRYSEMYS PICTA BELLII* IN BRITISH COLUMBIA

by

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Abstract

As biodiversity continues to be lost at an alarming rate, it has become increasingly important to resolve issues surrounding taxonomic uncertainty and how best to prioritize populations for conservation. Controversy can arise over whether populations merit conservation if their taxonomic status is unclear. Additionally, maintaining intraspecific genetic diversity is of particular importance for preserving evolutionary history and the potential for future adaptation. In order to effectively protect this diversity, species and units below the species level need to be defined. However, delineation of such units is subject to many challenges, with no one strategy applying universally across taxa. Using mitochondrial DNA sequence and microsatellite genotypic data, I examine population structure and demographic history of *Chrysemys picta bellii* (western painted turtles) in British Columbia, where it is a species-at-risk. I use this system to compare the application of evolutionarily significant unit and management unit criteria with Canadian designatable unit guidelines to determine appropriate conservation units. I find that that BC western painted turtles form a single evolutionarily significant unit, with each occupied site constituting a separate management unit. These findings contrast with the evidence for six discrete designatable units. Patterns of genetic variation in BC western painted turtles indicate that the conservation of each region is important to maintaining regional diversity and evolutionary novelty.

I also address the taxonomic uncertainty in *Chrysemys*, which is ambiguous due to questions regarding whether the four regional morphological variants warrant formal recognition. Despite using both mitochondrial and nuclear sequence data, I do not find genetic evidence to

conclusively resolve the taxonomic uncertainty in *Chrysemys*, however, there are indications that the two currently recognized species *Chrysemys dorsalis* and *Chrysemys picta* may be warranted.

Overall, this research presents the first detailed population genetic study for this species, which can be used to directly inform conservation prioritization of western painted turtles in BC. Moreover, the range-wide phylogeographic analyses is the most through genetic investigation of *Chrysemys* taxonomy to date, yet future research involving additional nuclear markers is still warranted.

Preface

Several individuals have contributed to Chapters 2 and 3 and Appendix A of this thesis. Manuscript versions of these will be co-authored when submitted for publication. The co-authors and I shared in the identification and design of this research. I have been primarily responsible for collecting the samples and data, data analysis and manuscript preparation.

A version of Chapter 2 has been submitted for publication:

> Jensen EL, Govindarajulu P, Russello MA (2013). When the shoe doesn't fit: applying conservation unit concepts to western painted turtles at their northern periphery

I collected most of the samples and all of the data used in the manuscript. I conducted all analyses and drafted the manuscript. Michael Russello oversaw data collection and analyses, and helped draft the manuscript. Purnima Govindarajulu guided sample collection and helped draft the manuscript.

Some of the lab work associated with Appendix A was carried out by Jeanette Madsen under my supervision as part of her undergraduate honors project, although I collected additional data and analyzed all data independently of her study.

The data presented in this thesis was collected according to the animal care protocol of the University of British Columbia Research Ethics Board (Animal Care Certificate # A11-0163) and sampling permit # VI11-71744 from the BC Ministry of Environment.

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A genetic assessment is not possible without DNA samples and my study would have far fewer of them if it weren't for the help of the Western Painted Turtle Working group. I would particularly like to thank Aimee Mitchell, Christian Engelstoft, David Stiles, Deanna Mactavish, Julie Steciw, Kristiina Ovaska, Michelle Evelyn, Nicole Basaraba, Melissa Tesche and Vanessa Kilburn for rolling up their sleeves and collecting samples. I would also like to thank Mark Hall, Kym Welstead and David Zehnder for helping me to identify sites.

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1 Chapter: INTRODUCTION

1.1 Conservation of biodiversity

The main goal of conservation biology is to preserve biodiversity, both in the number and variety of species present in an ecosystem, as well as the genetic diversity within those species (Frankham 2010). It is recognized that all three components of biological diversity (ecosystem, species and genetic) are important to protect (Convention on Biological Diversity 1992; McNeely et al. 1990). The maintenance of intraspecific genetic diversity is critical so that the process of evolution can continue without excessive constraint due to lack of natural variation (Frankel 1974; Mace et al. 2003). Explicitly incorporating genetic management into the protection of threatened species in the wild has emerged as one of the top priorities of conservation biology (Frankham 2010). However, the design and implementation of such strategies remains challenging due to several unresolved issues regarding the appropriate units for conservation. A fundamental issue is the continued lack of agreement over what constitutes a species. It is difficult to assess how many individuals there are in a species, and thereby determine conservation status (not at risk, threatened, endangered etc.), when it is unclear which groups of individuals have membership to that species (Agapow et al. 2004). Likewise, controversy persists regarding the designation of units below the species level for protecting intraspecific genetic diversity.

1.2 Species concepts

Since the time of Plato, philosophers and scientists have been trying to define groups of organisms, with the controversy over the reality and definition of species ongoing today

(Richards 2010; Wilkins 2009). Deciding upon a definition of species for conservation purposes is Frankham's (2010) second top priority for conservation biology, followed by deciding upon a definition of a conservation unit. There are many different species definitions, which according to Lucklow (1995) fall into two categories: mechanistic, where species are seen as units undergoing evolution, and historic, where species are seen as endpoints of evolution. Sites and Marshall (2003) reviewed both non-tree-based and tree-based methods for delineating species and concluded that it is not possible to generalize which method is best for more than one taxon.

There are two main species concepts upon which most conservation units are based. Under the biological species concept (BSC), intraspecific units that are evolutionarily important are possible, as species are defined as " groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1969). Indirect methods for determining a biological species involve looking for evidence of reciprocal monophyly of mitochondrial genes and concordance of gene-trees (Avise and Ball 1990). The main problem with the BSC is that the boundaries between species are largely untestable, especially in allopatry, and there are many cases where "species" can hybridize and produce viable offspring (Agapow et al. 2004). Also, it is arguable that methods for determining reciprocal monophyly are not valid below the species level, as phylogenetic analysis should be applied only to organisms related by a nested hierarchy, not those related by a pattern of ancestry and descent (see Goldstein et al. 2000 for a detailed discussion). Despite this controversy, the BSC remains one of the most popular and widely taught species concepts, and has many influential proponents (e.g. Avise and Ball 1990; Moritz 1994; Waples 1991).

The phylogenetic species concept (PSC) identifies species as "an irreducible cluster of organisms, within which there is a parental pattern of ancestry and descent, and which is diagnosably distinct from other such clusters" (Cracraft 1983). The methods for determining a phylogenetic species are based on finding the boundary between organisms that are related by a nested hierarchy and those that are related by a reticulating genealogy; all individuals within the reticulating genealogy are part of the phylogenetic species (Goldstein et al. 2000).This definition describes the minimal unit to which phylogenetic analysis can be legitimately applied (i.e. relationships between individuals are genealogical) that precludes the possibility for diagnosable, distinct, intraspecific units (Goldstein et al. 2000). If this species concept were accepted, then there would be no need for conservation units below the species level, as each species would be a single, cohesive evolutionary unit. This subdivision is the main fault that biologists have with the PSC: it describes too many entities. In a review by Agapow et al. (2004), application of the PSC resulted in 48% more species being named than other non-phylogenetic concepts. Frankham et al. (2012) caution that this over-splitting of taxa may result in an overall loss of biodiversity if small phylogenetic species of conservation concern suffer from inbreeding depression that could have been avoided through genetic rescue from other phylogenetic species. An advantage to the PSC is that species boundaries are hypotheses that are testable by the addition of more character data (Lucklow 1995).

Another controversy tied to species concepts is the subspecies problem. Since Linnaeus' time, subspecific designation has been used to describe individuals that share some morphological trait or variant (Haig et al. 2006). However, subspecies is the least agreed

upon unit in taxonomy, as there is no established definition and the concept is applied inconsistently across taxa and kingdoms (Haig et al. 2006). The PSC does not recognize subspecies, as species are an irreducible, diagnosable cluster of organisms (Cracraft 1983). It is generally understood that distinct and biologically significant populations should be lent conservation protection, but the semantics of what they should be called (species, subspecies, or conservation unit) and how to define such populations remains unclear.

1.3 Intra-specific conservation units

1.3.1 The Evolutionarily Significant Unit

Conservation may involve the recognition of units below the species level to which recovery goals and efforts are applied. These units were typically based on the taxonomic level of subspecies, until Ryder (1986) published a paper describing the fundamental problems with basing conservation on taxonomic units that do not necessarily have an evolutionary basis. The concept of an evolutionarily significant unit (ESU) was proposed as a way to identify populations for conservation that were truly reflective of the evolutionary trajectory of the species (Ryder 1986), as opposed to using the subjective unit of subspecies. Zoo systems were being overwhelmed by the vast number of named subspecies apparently in need of *ex situ* conservation, while many of the subspecies were of uncertain validity. The ESU concept stemmed out of the need for zoo biologists to decide for themselves which lineages represent genetic variation significant to the present and future of the species and are most important to preserve within the limitations of *ex situ* conservation (Ryder 1986).

Ryder (1986) acknowledged that identifying ESUs would be difficult and recommended using a combination of natural history, phylogeography, and genetic analysis to delineate these intraspecific units. Since then, both the criteria for identifying and the definition of an ESU have undergone many interpretations by different authors. Waples (1991) defined a "species" under the U.S. Endangered Species Act as a population segment meeting two criteria: reproductive isolation from other conspecific units, and representing an important part of the evolutionary legacy of the species (either unique ecologically or genetically). His criteria for determining if a unit is evolutionarily important include: genetic distinctiveness, occupation of unique habitat, possession of unique adaptations to the environment and significant loss to the ecological or genetic diversity of the species if that unit became extinct. Dizon et al. (1992) wrote that ESUs must be evaluated based on their adaptive genetic uniqueness, and that behaviour, morphology and geographic location could be used as proxies for genetic adaptation. Unlike previous definitions, Vogler and Desalle (1994) advocated for the use of character data for cladistics analysis and population aggregation analysis to evaluate ESUs and turn them into testable hypotheses. The most broadly applied ESU definition to date is that of Moritz (1994). In this case, ESUs are those that are reciprocally monophyletic for mitochondrial haplotypes and show statistically significant divergence of allele frequencies at nuclear loci. This definition, although widely applied, is critiqued as being highly restrictive and incompatible with the phylogenetic species concept (Goldstein et al. 2000).

Other concepts attempt to add flexibility to the ESU concept. Frazer and Bernatchez (2001) attempted to reconcile the proposals of other authors by offering adaptive evolutionary

conservation (AEC) as a compromise, because they did not think that a single, rigid universal definition of an ESU will be appropriate in all situations. Under AEC, a conservation unit should be "a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level of the species" (Fraser and Bernatchez 2001). They felt that any criteria, including adaptive divergence and historical isolation, that give evidence for lineage sorting as a result of restricted gene flow could be used to delineate biologically meaningful ESUs. In contrast, Crandall et al. (2000) argued against the ESU/not-ESU dichotomy of other authors' definitions. They proposed using a continuum of classifications describing how exchangeable individuals are between populations, taking into account ecological, genetic, recent and historical exchangeability and scoring each category (Crandall et al. 2000)

A commonality among many of the versions of the ESU is the need for evidence of genetic divergence between units. There is debate, however, about the roles that neutral versus adaptive markers should play. Some authors maintain that neutral loci can be used to reliably estimate patterns of reproductive isolation and the demographic history of populations (Allendorf et al. 2010; Miller et al. 2010), which are the mechanisms that result in divergence. Other authors argue that patterns in neutral maker variation are not indicative of underlying adaptive genomic variation (Kohn et al. 2006; Ouborg et al. 2009; Reed and Frankham 2001; Sgro et al. 2011) and that units that appear differentiated at neutral loci are not necessarily significantly evolutionarily distinct. The term "partial-ESU" was put forth by De Guia and Saitoh (2006) to describe a unit that was designated based on either neutral or adaptive markers but not both. De Guia and Saitoh (2006) argued that both types of marker

need to be used to accurately recognize the status of a unit, and full ESU rank should be reserved for units supported by both types of evidence. Genomic information that would allow the identification of adaptive divergence is not currently available for most organisms of conservation concern, however, recent technological advances, such as the development of the double digest RAD sequencing pipeline (Peterson et al. 2012), may allow the development of adaptive genomic markers in non-model organisms. There are no clear guidelines about how such information could be used to delineate conservation units, however several authors have begun to make proposals (e.g. Bonin et al. 2007; Funk et al. 2012).

1.3.2 Policy based conservation units

In the face of rapid declines in many species and the lack of information on genetic and phylogenetic relatedness, conservation units are sometimes designated based on applied management imperatives. Although based on scientific principles, the designation of these conservation units is not necessarily based on species-specific genetic or phylogenetic information. The geopolitical unit (GU) was proposed to lend protection to populations within a particular political region, regardless of the status of conspecific populations in other political regions and independent of morphological, genetic or reproductive criteria (Karl and Bowen 1999). In Canada, the Species at Risk Act (SARA) lends protection to 'distinct populations of wildlife', which are identified by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) according to the designatable unit (DU) concept. COSEWIC (2008) may delineate DUs that are named subspecies or populations that are discrete and evolutionarily or ecologically significant without formal taxonomic distinction. Evidence of

population discreetness could include morphology, life history or behavioural traits and genetic evidence based on neutral genetic markers (COSEWIC 2004b). The significance of a population is assessed based on phylogenetic divergence, occupation of unique ecological settings resulting in local adaptation, evidence that it is the last surviving population within the species' historical range (although it may be introduced elsewhere) and evidence that the loss of that population would result in a gap in the range of the species in Canada (COSEWIC 2004b). This Canadian policy is similar to that lending protection to distinct population segments under the Endangered Species Act in the United States of America, although there are differences in the way that the policies are applied (US Fish and Wildlife Service and National Oceanic and Atmospheric Administration 1996).

1.4 Other issues in conservation

The conservation of wide ranging species requires special consideration because, across a species' distribution, populations at the margins of the range may represent unique pockets of genetic diversity and ecological characteristics (Gaston 2003). This pattern can be due to reduced gene flow with the core of the range and small population sizes resulting in elevated levels of differentiation and disparate selection pressures (Lesica and Allendorf 1995). Conserving and maintaining healthy peripheral populations may be important for the long-term persistence of species, as it has been found that the ranges of many endangered mammals have collapsed not into the core of their historical ranges, but in fact into their periphery (Lomolino and Channell 1995). In the northern hemisphere, the trend of collapse tends to be into the northwestern periphery of the range (Channell and Lomolino 2000). This

pattern goes against some of the most common recommendations for conservation, which put protecting core populations at a higher priority than peripheral ones (Griffith et al. 1989).

The current biodiversity crisis is affecting some taxa out of proportion to others. Turtles are one such group, with at least 47% of the 330 currently described terrestrial and freshwater species threatened with extinction (Rhodin et al. 2010). As of 2012, six out of 12 species of terrestrial and freshwater turtle with the northern periphery of their range in Canada are listed as threatened or endangered by COSEWIC (Kiester and Olson 2011) with a further two species of special concern and one species extirpated. The major causes of turtles' decline globally are habitat destruction, road mortality, pollution and over-exploitation for human use in traditional medicine, as pets and for meat (Alacs et al. 2007; Gibbon et al. 2000; Kiester and Olson 2011). Climate change is also seen as a looming threat for the turtle species that have temperature-dependent sex determination (Gibbon et al. 2000; Telemeco et al. 2013).

Turtles are one of the most ancient extant lineages on the animal tree of life, but certain life history characteristics that may have helped them survive mass extinctions and global change in the past might now be working against them. Turtles are long lived with advanced age at sexual maturity often resulting in overlapping generations, which can have serious implications for inbreeding levels in small populations (Alacs et al. 2007). They also have mutation rates eight times slower than other vertebrates within the mitochondrial genome (Avise et al. 1992). With these factors in mind, it is imperative that turtle conservation

strategies are designed to preserve intraspecific genetic diversity because diversity, once lost, is not likely to regenerate quickly (Alacs et al. 2007).

1.5 The western painted turtle

1.5.1 *Chrysemys* taxonomy

Chrysemys (Schneider 1783) is a genus within the family Emydidae that is experiencing ongoing taxonomic revisions due to debate as regards to the status of four formally described morphotypes. Currently, the Society for the Study of Amphibians and Reptiles recognises two species: *Chrysemys dorsalis* and *Chrysemys picta*, the latter of which is composed of three subspecies: *C. p. picta*, *C. p. bellii* and *C. p. marginata* (Crother 2012). These designations are largely based on a study that looked at sequences of the mitochondrial control region (CR) of individuals across the United States, and showed *C. dorsalis* as a different evolutionary lineage from the other three morphotypes (Starkey et al. 2003). Other taxonomists disagree with the elevation of *C. dorsalis* to the species level, and argue that it does not have either geographic or reproductive isolation from the subspecies within *C. picta* (Ernst and Lovich 2009). Interbreeding and hybridization are common wherever the ranges of the morphotypes overlap (e.g. Weller et al. 2010) to the extent that some speculate that a continuously shared gene pool may exist over the entire range of *Chrysemys* (Ernst and Lovich 2009).

1.5.2 Chrysemys picta bellii

Chrysemys picta bellii (Gray 1831), commonly known as the western painted turtle, is the largest and most distinctively coloured subspecies. The range of *C. p. bellii* extends across the southernmost part of Canada west of the Great Lakes to British Columbia, and in the United States as far south as Missouri continuously and into Mexico as isolated populations (Fritz and Havas 2007). There are currently no accurate range maps available from reputable sources for *Chrysemys*.

1.5.3 British Columbian western painted turtles

In 2006, COSEWIC reviewed the status of *C. p. bellii* in Canada. Three DUs were identified: Pacific Coastal population (Endangered), Intermountain-Rocky Mountain population (Special Concern), and Prairie/Western Boreal-Canadian Shield population (Not at Risk) (Figure 1.1). In the absence of species specific information for herptofauna, COSEWIC recognizes eight distinct faunal provinces (Figure 1.1) and these faunal provinces are then used to delineate DUs for species, as was done for the western painted turtle. Both the Pacific Coastal and Intermountain-Rocky Mountain DUs occur in BC and encompass three faunal provinces (COSEWIC 2006). As these conservation units were delimited based on the generic faunal provinces, it is unclear whether these three DUs reflect distinct genetic units.

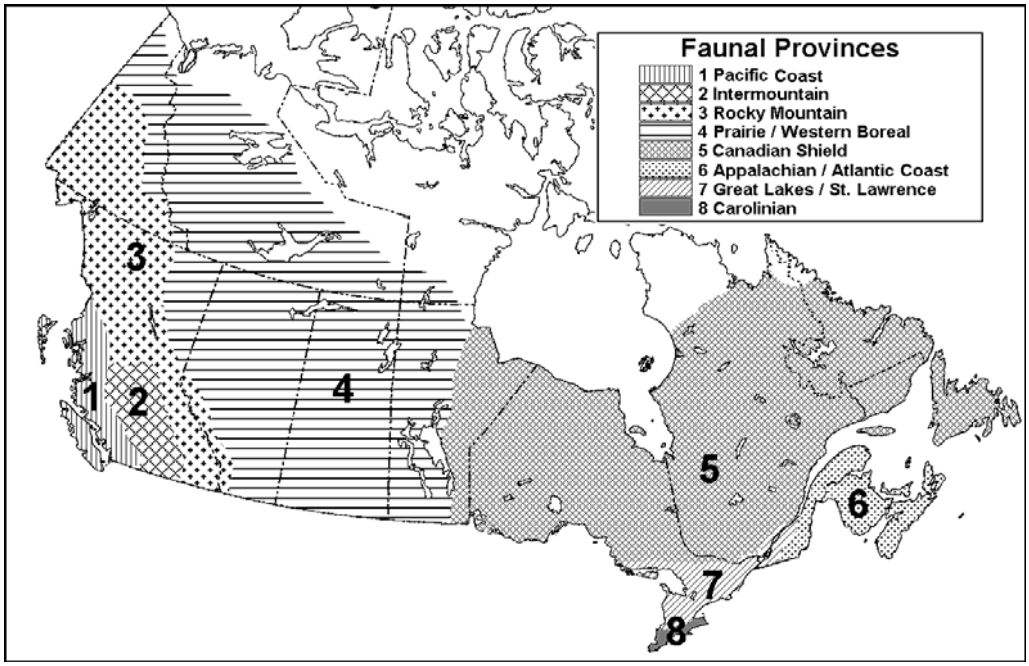


Figure 1.1 Map of the faunal provinces in Canada for amphibians and reptiles, which corresponds to the designatable units for *Chrysemys picta bellii*.
Reproduced from COSEWIC (2006)

The endangered status of the Pacific Coastal population is particularly controversial because there is anecdotal evidence that at least some of these populations or some individuals within these populations have been introduced as a result of humans releasing turtles from elsewhere in their range (COSEWIC 2006). If this is the case, then their conservation value is questionable. Records of western painted turtles on Vancouver Island date back to the 1920s (COSEWIC 2006), so it is possible that the Pacific Coastal populations are native, but that turtles there have always been rare. Genetic comparison of the relatedness of Pacific Coastal western painted turtles to other populations could help determine whether they are native or have been introduced over time to that region.

C. p. bellii is one of only two species of freshwater turtle native to British Columbia. The other species, *Emmys marmorata marmorata* (Northern Pacific Pond Turtle), has been extirpated from Canada (COSEWIC 2004a). Red eared sliders (*Trachemys scripta*), an introduced species, are becoming increasingly common in ponds, lakes and streams in BC, with robust populations in the Fraser Valley and elsewhere in Western North America as a result of pets being released (Bunnell 2005). The presence of a non-native potential competitor is seen as a threat to western painted turtles in BC (COSEWIC 2006).

Of growing concern in the Lower Mainland of BC is the appearance of putative introduced individuals of the other subspecies of *C. picta*. Hybridization is a common occurrence where the ranges of the different subspecies naturally overlap (Weller et al. 2010); however, the presence of other subspecies in B.C. is due to human interference, likely via the release of unwanted pets. Interbreeding between native and introduced individuals would result in the introduction of alleles that could be detrimental to the local populations if those alleles are maladaptive to the local conditions (Templeton 1986). Also, genetic contamination could affect the conservation value of introgressed populations (Allendorf et al. 2001).

Due to clinal variation in morphology within the range of each subspecies (Ultsch et al. 2001), it is difficult to determine whether individuals are hybrids based solely on morphology. Hybridization resulting in intermediate morphology is common wherever the ranges of subspecies overlap (Weller et al. 2010). Inability to distinguish hybrids based on morphology means that genetic methods will need to be used to detect hybrids reliably and determine levels of introgression in a population.

1.6 Thesis objectives

The primary goal of this thesis is to carry out an explicit genetic assessment of western painted turtles across BC to reconstruct their population history, phylogenetic placement and conservation status. In chapter 2, I use mitochondrial DNA haplotypic and microsatellite genotypic data to assess the extent and distribution of genetic variation within and among BC western painted turtle populations. I further use this information to evaluate the current DUs and examine what the conservation priorities in BC would be under the conservation unit criteria of Moritz (1994) and COSEWIC (2008) and reconstruct demographic history. In chapter 3, I place BC western painted turtles in the context of the broader species distribution using mitochondrial and nuclear gene sequence data and examine the phylogenetic relationships among the four morphological types or subspecies within *Chrysemys* to further investigate taxonomic uncertainty and paleogeographic hypotheses within the group.

2 Chapter: APPLYING CONSERVATION UNIT CONCEPTS TO WESTERN PAINTED TURTLES AT THEIR NORTHERN PERIPHERY

2.1 Background

In the face of the on-going biological diversity crisis, an ever increasing number of species are considered threatened with extinction (Vié et al. 2009). As biodiversity continues to be lost, strategies for prioritizing populations for conservation have become increasingly important (Avise 2005). The maintenance of intraspecific genetic diversity is of particular importance for preserving both evolutionary history and the potential for future adaptation (Frankham 2010; Frankel 1974). In order to protect this diversity effectively, units below the species level need to be defined, and should be the level at which recovery goals and efforts are typically applied. Incorporating genetic management into the protection of threatened species in this way is one of the top priorities of conservation biology (Frankham 2010).

The delineation of units below the species level for conservation purposes is subject to many challenges, with no one strategy that applies universally across taxa and systems. Chief among these difficulties is deciding what criteria (genetic, geographic, political etc.) should be used to assess whether units are sufficiently diverged to warrant separate management and which genetic markers, if any, should be used in this determination. Subspecies constitute a controversial unit in taxonomy (Haig et al. 2006), and have been previously deemphasized as an appropriate unit for conservation by Ryder (1986) in favour of non-taxonomic methods. Since that time, a primary focus has been on delineating evolutionarily significant units (ESU), originally defined as real biological entities that possess a significant evolutionary

lineage (Ryder 1986). The procedures for delineating such units, however, have undergone many different interpretations by various authors since it was first proposed (Crandall et al. 2000; Dizon et al. 1992; Fraser and Bernatchez 2001; Moritz 1994; Vogler and Desalle 1994; Waples 1991).

The ESU *sensu* Moritz (1994) is the most widely applied version, principally because it explicitly uses the genetic criteria of reciprocal monophyly of mitochondrial DNA variation and significant allele frequency divergence at nuclear loci to define units for conservation. Units defined in this way consist of groups of populations that have been historically isolated from other such groups of populations. Within an ESU, populations that have diverged at allele frequencies are also recognized as significant for conservation as they are connected by low levels of gene flow and are functionally independent. These intra-ESU groups are referred to by Moritz (1994) as management units (MU) and are the level at which demographic and population monitoring should take place.

The ESU is not the only conservation unit concept, with many others having been defined based on their use of genetic and ecological data, type of genetic marker (nuclear versus organellar; neutral versus putatively adaptive loci) and level of emphasis along the spectrum from populations to species (Karl and Bowen 1999; Wood and Gross 2008). Among these other methods are a class of concepts that recognize that, in the face of rapid declines in many species and the lack of information on genetic and phylogenetic relatedness, conservation units must sometimes be designated based on management imperatives. In Canada, the Species at Risk Act (SARA) lends protection to ‘distinct populations of

wildlife', which are identified by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) according to the designatable unit (DU) concept. COSEWIC may delineate DUs as named subspecies or populations that are discrete and evolutionarily or ecologically significant even if these units lack formal taxonomic distinction (COSEWIC 2008). Evidence for population discreteness may include genetics, geographic disjunction, morphology, life history and/or behaviour (COSEWIC 2008). The significance of a population is assessed based on phylogenetic divergence, occupation of unique ecological settings resulting in local adaptation, evidence that it is the last surviving population within the species' historical range (although it may be introduced elsewhere), and/or evidence that the loss of that population would result in a gap in the range of the species in Canada (COSEWIC 2008). This Canadian policy is similar to that lending protection to distinct population segments under the Endangered Species Act in the United States of America, although there are important differences in the way that the policies are applied (US Fish and Wildlife Service and National Oceanic and Atmospheric Administration 1996).

The conservation of wide ranging species requires special consideration because, across a species' distribution, populations at the margins of the range may represent unique pockets of genetic diversity and ecological characteristics (Gaston 2003). This pattern can be due to reduced gene flow with the core of the range and small population sizes resulting in elevated levels of differentiation and disparate selection pressures (Lesica and Allendorf 1995). Conserving and maintaining healthy peripheral populations may be important for the long-term persistence of species, as it has been found that the ranges of many endangered mammals have collapsed, not into the core of their historical ranges, but towards their

periphery (Lomolino and Channell 1995). In the northern hemisphere, the trend of collapse tends to be into the northwestern periphery of the range (Channell and Lomolino 2000). This pattern goes against some of the most common recommendations for conservation, which emphasize protection of core populations to a greater degree than peripheral ones (Griffith et al. 1989).

The painted turtle *Chrysemys picta* is a wide spread species, with a range that is extensive throughout the United States of America and the southernmost portions of Canada (Ernst and Lovich 2009). The western painted turtle (*Chrysemys picta bellii*) is one of four recognized subspecies, and is the only native member of the Emydidae extant in western Canada. In 2006, COSEWIC assessed the status of western painted turtles in Canada due to concerns that habitat loss was causing major population declines in parts of British Columba (BC). That process identified three designatable units (DU) of western painted turtle in Canada, two of which occur within the political borders of BC: the Pacific coastal population (Endangered) and the Intermountain-Rocky Mountain population (Special Concern) (COSEWIC 2006). The third DU is listed as Not at Risk, and encompasses the rest of the range of the western painted turtle within Canada, spanning from Alberta to Ontario. When population genetic evidence is not available, COSEWIC may delineate DUs based on generic faunal provinces, as was the case with western painted turtles in 2006. It is unclear whether these three DUs reflect distinct genetic units.

In this study, I used mitochondrial DNA haplotypic and microsatellite genotypic data to assess the extent and distribution of genetic variation within and among BC western painted

turtle populations. Observed patterns were used to reconstruct demographic history, infer population connectivity, and examine current designatable units in light of the genetic evidence to inform management actions at the northern periphery of the species' range.

2.2 Materials and methods

2.2.1 Sampling

Samples were collected from 475 individuals of western painted turtle from 25 sites within BC during 2011-2012 (Figure 2.1, Table 2.1, Table B.1). An additional 73 individuals were sampled from four more sites but later excluded from this study (see Appendix A and B). An individual site consisted of a single discrete pond or lake, except in two cases, where neighbouring lakes (Baird and Hidden Lake, <1.3 km apart) or lakes on a small island (Texada Island: Case, Emily, Capsheaf and Priest Lakes) were considered a single site (see Appendix B). These sites span the known geographic range of the species in BC (Figure 2.1). Turtles were caught using hoop traps baited with canned sardines or by hand using a dip net. At each site, a blood sample (~100µl) from six to 37 individuals (Table 2.1) was collected from either the dorsal coccygeal vein or brachial artery. Blood was stored in tubes containing a buffer solution (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS; Longmire et al. 1997) and stored at 4 ˚C until analysis. At some sites, tissue samples were excised from road-killed individuals and stored in 100% ethanol or shell samples were clipped from the margin of scutes on the upper plastron and stored in paper envelopes or dry tubes. All samples were collected in accordance with University of British Columbia Animal

Care Certificate # A11-0163 and sampling permit # VI11-71744 from the BC Ministry of Environment.

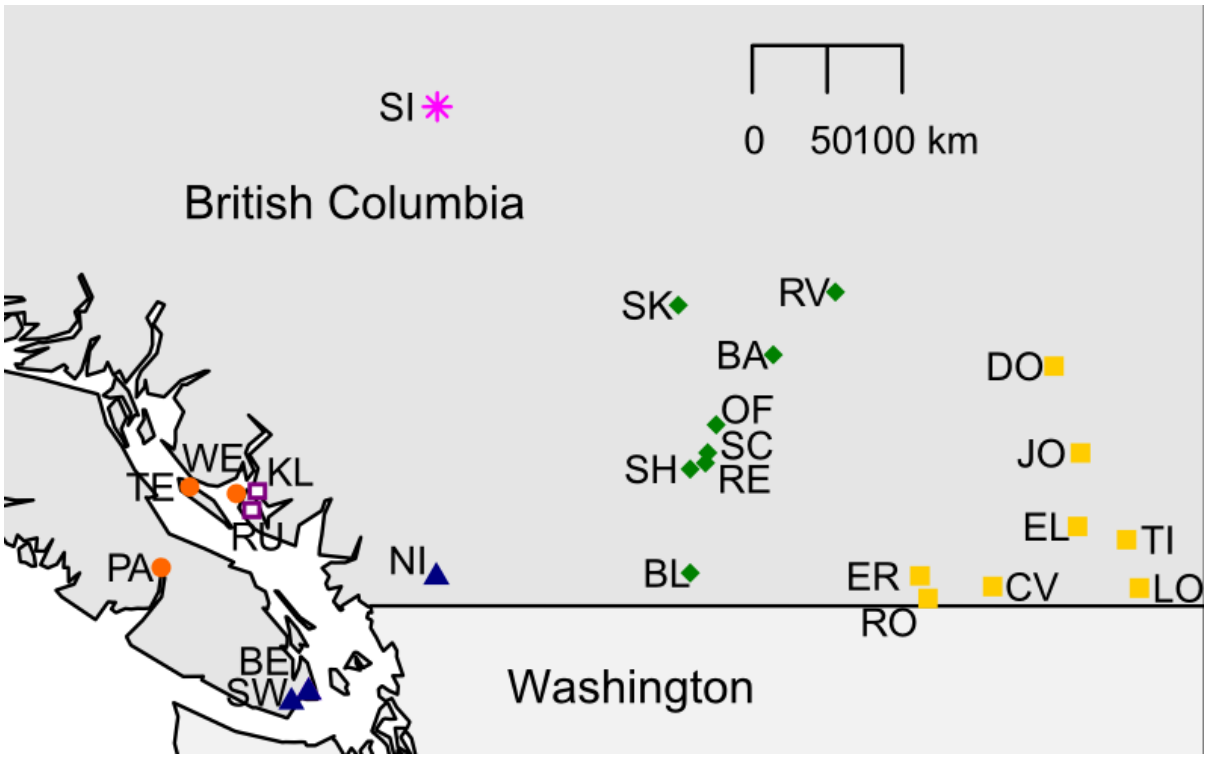


Figure 2.1 Map of study area indicating western painted turtle sampling locations within British Columbia

Site acronyms follow Table 2.1. Colours and symbols indicate genetic clusters as follows: Sunshine coast-Gulf Island-Mid-Vancouver Island (SGV; orange circle), Sunshine coast (purple open square), South coast (blue triangle), Cariboo (pink star), Thompson-Okanagan (green diamond), Kootenay (yellow square). The heavy line represents the border between British Columbia, Canada and Washington, USA

Table 2.1 Sampling locations and sample sizes of western painted turtle collected in BC, including estimates of within-site genetic variation and demographic history

Site	Site Name	Genetic Cluster	*N*	A_R	H_e	H_o	P_A	F_{IS}	LR-R
BA	Baird Lake	Thompson-Okanagan	23	5.22	0.69	0.72	0	-0.05(-0.16 - 0.01)	0.16
BE	Beaver Lake	South coast	18	5.89	0.76	0.81	4	-0.07(-0.22 - 0.0)	0.25
BL	Burnell Lake	Thompson-Okanagan	20	6.22	0.75	0.66	0	0.13(0.02 - 0.18)	0.08
CV	Creston Valley Wildlife Management Area	Kootenay	15	6.67	0.75	0.77	2	-0.03(-0.15 - 0.01)	0.08
DO	Dorothy Lake	Kootenay	14	5.67	0.76	0.84	0	-0.11 (-0.25 - -0.06)	0.12
EL	Elizabeth Lake	Kootenay	18	6.67	0.76	0.71	0	0.07 (-0.05 - 0.13)	0.08
ER	Erie Lake	Kootenay	6	4.78	0.75	0.70	0	0.07 (-0.3 - 0.14)	0.16
JO	Johnson Lake	Kootenay	21	6.11	0.69	0.66	0	0.04 (-0.07 - 0.1)	0.15
KL	Klein Lake	Sunshine Coast	15	2.89	0.51	0.61	0	-0.21 (-0.36 - -0.13)	0.46
LO	Loon Lake	Kootenay	25	7.00	0.76	0.72	2	0.06 (-0.03 - 0.09)	0.12
NI	Nicomen Slough	South coast	21	4.44	0.65	0.74	4	-0.16 (-0.26 - -0.11)	0.34
OF	Fipke Lake near Oyama	Thompson-Okanagan	21	6.56	0.75	0.74	0	0.0 (-0.1 - 0.04)	0.09
PA	Port Alberni	SGV	13	3.89	0.57	0.56	1	0.02 (-0.16 - 0.1)	0.27
RE	Redlich Pond	Thompson-Okanagan	7	5.00	0.72	0.63	1	0.12 (-0.17 - 0.22)	0.02
RV	Revelstoke Marsh	Thompson-Okanagan	20	6.44	0.77	0.76	2	0.01 (-0.1 - 0.05)	0.14
RO	Rosebud Lake	Kootenay	25	5.44	0.67	0.65	1	0.04 (-0.06 - 0.09)	0.2
RU	Ruby Lake Lagoon	Sunshine Coast	18	4.44	0.61	0.63	0	-0.03 (-0.16 - 0.05)	0.26
SI	Scout Island Nature Centre	Cariboo	24	4.22	0.65	0.70	0	-0.07 (-0.18 - 0.01)	0.34
SC	Stephen Coyote Regional Park	Thompson-Okanagan	37	6.44	0.74	0.69	0	0.06 (-0.03 - 0.11)	0.11
SH	Shannon Lake	Thompson-Okanagan	19	5.56	0.71	0.71	0	-0.01 (-0.16 - 0.09)	0.13
SK	Skmana Lake	Thompson-Okanagan	20	4.56	0.61	0.61	0	0.0 (-0.13 - 0.08)	0.2
SW	Swan Lake	South coast	10	3.67	0.60	0.72	0	-0.22 (-0.47 - -0.17)	0.28
TE	Texada Island	SGV	17	3.78	0.51	0.45	0	0.13 (-0.06 - 0.23)	0.32
TI	Tie Lake	Kootenay	21	6.00	0.72	0.68	0	0.05 (-0.06 - 0.12)	0.13
WE	West Lake	SGV	27	3.44	0.55	0.56	0	-0.01 (-0.16 - 0.08)	0.45
Total/ Average			**475**	**5.20**	**0.68**	**0.68**	**17**	**-0.17**	**0.2**

N number of individuals, A_R mean number of alleles, *He* expected heterozygosity, *Ho* observed heterozygosity, P_A private alleles, F_{IS} inbreeding coefficient with 95 % confidence interval, *LR-R* Lynch Ritland relatedness estimator

2.2.2 Data collection

DNA was extracted from blood samples using the Nucleospin QuickBlood kit (Macherey-Nagel) following manufacturer's protocols. DNA was extracted from tissue samples using the Nucleospin Tissue kit (Macherey-Nagel) following manufacturer's protocols, except in the case of shell samples, where the initial incubation with Proteinase K was carried out at 45˚C overnight (~16 hours).

For a representative sample from each site (1-3 individuals), a 671 base pair fragment of the mitochondrial DNA (mtDNA) control region (CR) was amplified as a single fragment using DES1 (Starkey et al. 2003) and Cp_CRExt primer (5'-GCTCTCGGATTTAGGGGTTT-3´). Polymerase chain reactions (PCR) were carried out on an ABI Veriti thermal cycler in 25 µl reactions containing: ~20-40 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 7.5µg bovine serum albumin, 0.4µM each primer and 0.5 U Kapa Taq DNA Polymerase (Kapa Biosystems). Cycling conditions consisted of 95˚C for 2 minutes, 35 cycles of 95˚C for 30 seconds, 55˚C for 1 minute, 72˚C for 2 minutes, and a final extension at 72˚C for 10 minutes. The sequencing reactions were performed using ABI BigDye v3.1 Terminator chemistry and sequences were run on an Applied Biosystems 3130XL DNA automated sequencer. Sequences were visualized and edited using Sequencher 5.0 (Gene Codes Corporation).

Nuclear genotypic data were collected for all 475 samples at 10 microsatellite loci (Table B.2) (Gonçalves da Silva et al. 2009; Hauswaldt and Glenn 2003; King and Julian 2004; Pearse et al. 2001). All forward primers were 5'- tailed with an M13 sequence and used in

combination with an M13 primer of the same sequence 5'-labeled with one of four fluorescent dyes (6-FAM, VIC, NED, PET) to facilitate automated genotyping (Schuelke 2000). PCRs were carried out on an ABI Veriti thermal cycler in 12.5 µl reactions containing: ~20-40 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 7.5µg bovine serum albumin, 0.04 µM of the M13-tailed forward primer, 0.4 µM each of the reverse primer and the M13 fluorescent dye-labeled primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Reaction conditions for all primer sets were optimized using a 'touchdown' cycling program that consisted of: 95˚C for 10 min; 35 cycles of 95˚C for 30 s, annealing for 30 s, and 72˚C for 45 s; and a final step of 72˚C for 7 min. We used two versions of the annealing step in the 'touchdown' program. The annealing temperature decreased 1˚C each cycle from 59˚C or 55˚C until it reached 51˚C or 45˚C, respectively, at which point the remaining cycles continued with a 51˚C or 45˚C annealing temperature. Loci were co-loaded and run on an Applied Biosystems 3130XL DNA automated sequencer. All alleles were scored using bins in the software Genemapper 4.0 (Applied Biosystems, Inc.). Bins were evaluated with the program Tandem (Matschiner and Salzburger 2009), and all allele calls were manually verified.

2.2.3 Haplotypic variation and network analysis

In addition to the mtDNA sequence data collected for my study in BC, exemplar sequences from across the range of the western painted turtle were taken from GenBank (Starkey et al. 2003) (see Table B.3 for accession numbers). The mtDNA sequences were aligned in Geneious (Biomatters Ltd.) using Geneious Aligner (default settings) and a haplotype

network was generated using statistical parsimony, as implemented in TCS (Clement et al. 2000).

2.2.4 Genotypic variation and population differentiation

The genotypic data set was examined for the presence of null alleles using MICROCHECKER (Van Oosterhout et al. 2004). To test whether any loci deviated from neutral expectations, we conducted F_{ST} outlier detection tests as implemented in LOSITAN (Antao et al. 2008). This method creates an expected distribution of the relationship between F_{ST} and expected heterozygosity (H_e) with neutral markers, and then uses that distribution to identify loci that have significantly higher or lower F_{ST} compared to neutral expectations. Outlier loci are candidates for exhibiting locus-specific effects, including due to selection. Deviation from Hardy-Weinberg equilibrium (HWE) was assessed using exact tests, as implemented in GENEPOP 3.3 (Raymond and Rousset 1995; Rousset 2008). Linkage disequilibrium (LD) was investigated for all pairs of loci using GENEPOP 3.3 (Raymond and Rousset 1995; Rousset 2008). Type I error rates for tests of linkage disequilibrium and departure from HWE were corrected for multiple comparisons using the sequential Bonferroni procedure (Rice 1989).

Allelic diversity, observed (H_o) and expected heterozygosity (H_e) were calculated at each locus for each site using ARLEQUIN (Excoffier et al. 2005). A list of private alleles was tabulated using GenAlEx (Peakall and Smouse 2006).

Levels of genetic differentiation among sites were estimated by pairwise site comparisons of θ (Weir and Cockerham 1984) as calculated in FSTAT (Goudet 2001). Multiple comparisons were corrected for using the false discovery rate correction (Benjamini and Hochberg 1995), as advocated by Narum (2006) for use in conservation genetic studies. A matrix of the geographic distance between sites was created using the Geographic Distance Matrix Generator (Ersts 2012). To test the relationship between genetic differentiation and geographic distance, a Mantel test was performed between the matrices of θ values and geographic distance using the Isolation by Distance Web Service (Jensen et al. 2005). To further examine isolation by distance patterns, a non-stationary genetic friction map was developed to display geographic areas with a relatively larger genetic difference per unit of geographic distance using a similarity matrix (1-θ), 4 simulated neighbours at a distance of 0.1 and 100 posterior replicates as implemented in LOCALDIFF (Duforet-Frebourg and Blum 2012). The directions and magnitudes of contemporary migration rates among sites were estimated using a non-equilibrium Bayesian method implemented in BAYESASS version 3 (Wilson and Rannala 2003). The program run length was 10,000,000 MCMC replicates after a burn-in period of 1,000,000, sampling the chain every 100 iterations. Consistency of the results was assured by running the program five times using different random seeds and monitoring the output as visualized in TRACER (Rambaut and Drummond 2007).

To determine the number of discrete genetic units within the data set, the Bayesian method of Pritchard et al. (2000) was used as implemented in STRUCTURE 2.3.4. Run length was set to 500,000 MCMC replicates after a burn-in period of 250,000 using correlated allele

frequencies under a straight admixture model as well as using the LOCPRIOR option, the latter of which uses sampling locations as prior information to assist in clustering. The most likely number of clusters was determined by varying the number of clusters (K) from 1 to 25 with ten iterations per value of K and calculating ΔK (Evanno et al. 2005) as implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2011). To evaluate whether the genetic clusters identified through the STRUCTURE analysis conform to Hardy-Weinberg Equilibrium, an exact test for heterozygote deficit was implemented in GENEPOP (Rousset 2008; Raymond and Rousset 1995). Clusters that showed heterozygote deficit indicative of a potential Wahlund effect (Wahlund 1928) were separately analysed in STRUCTURE to determine if further substructure existed.

Analyses of molecular variance (AMOVAs) were performed using ARLEQUIN (Excoffier et al. 2005) for a range of grouping strategies including: 1) current DUs; 2) faunal provinces; 3) ecological drainage units of BC (EAU BC, accessed through iMap BC); and 4) clusters identified by STRUCTURE (see Table B.1 for further details). The faunal provinces were chosen as an *a priori* grouping strategy because they were used when delineating the DUs. The ecological drainage units were chosen as an *a priori* grouping strategy as they are an alternative strategy to the faunal provinces, both of which are biogeographically based.

2.2.5 Demographic history

The effective population size (N_e) of each STRUCTURE cluster was estimated using LDNe (Waples and Do 2008), which implements the bias correction of Waples (2006). Random

mating was assumed and alleles with frequencies lower than 0.02 were excluded from analyses.

Genetic signatures of demographic contraction were assessed using the heterozygote excess test and the mode-shift test, both implemented in BOTTLENECK 1.2.02 (Piry et al. 1999) and the *M*-ratio test using M_P_VAL.exe and critical_M.exe (Garza and Williamson 2001). For the heterozygote excess test, 1000 iterations were used with the Wilcoxon test under the two phase model with 90% stepwise mutations. For the *M*-ratio we calculated θ ($\theta=4N_e\mu$) using a mutation rate (μ) of $1.5x10^{-4}$ and a pre-bottleneck N_e of 50, 500 and 1500, resulting in θ = 0.03, 0.3 and 0.9, respectively. Multiple values of θ were used to assess how robust conclusions were to permutation of that parameter. We used 3.5 base steps for multi-step mutations, and the amount of single step mutations, P_s, was 0.1.

Genetic signatures of post-glacial expansion into BC were investigated using two methods that infer population expansion based on microsatellite gene genealogies: a within-locus *k* test and an inter-locus *g* test (Reich et al. 1999), using the kgtests EXCEL macro (Bilgin 2007). The significance table in Reich et al. (1999) was used to assess the *g* values.

Relatedness and inbreeding coefficients were estimated among individuals sampled at a site. Pairwise relatedness was calculated within and among sites according to the method of Lynch and Ritland (1999) in COANCESTRY (Wang 2011). The inbreeding coefficient, F_{IS}, was calculated for each sampling site as implemented in GENETIX (Belkhir et al. 2004).

2.3 Results

2.3.1 Haplotypic variation

The mtDNA CR fragment was sequenced in 72 individuals from across BC. One major (83%) and three low frequency (7%, 7%, 3%, respectively) haplotypes were identified, each differing by a single character from the major haplotype (Figure 2.2). Two of these haplotypes were unique to BC as determined by a comparative analysis with the 90 sequences from across the range of the western painted turtle published in Starkey et al (2003).

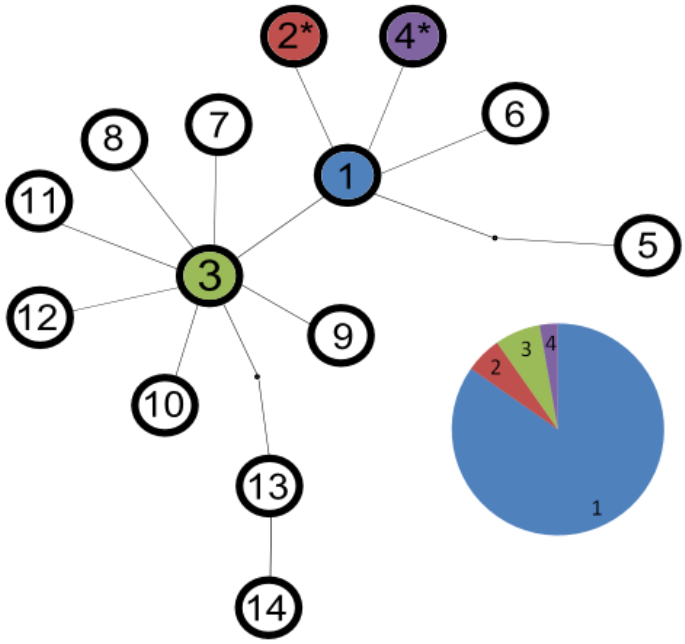


Figure 2.2. Haplotype network based on partial mitochondrial control region (671 bp) of western painted turtles from British Columbia, Canada and across the US including Washington, Colorado, New Mexico, Minnesota, Kansas, Wisconsin, South Dakota, Nebraska, North Dakota, Montana, Illinois, Missouri and Iowa.

Each haplotype is represented by a circle and numerical identifier. The circles filled in colour are those present in BC, with an asterisk indicating haplotypes unique to BC. Inset pie chart represents the proportion of each haplotype recovered. Unsampled intermediate haplotypes are represented by a solid circle

2.3.2 Genotypic variation and population differentiation

The Cp2 locus had null alleles and was removed from all further analyses. Other loci (GmuD21, GmuD28, GmuD62, GT108, Terp2 and Terp7) were also found to have null alleles in a small proportion of sites (n=1-9 out of 25), with three sites having two loci exhibiting null alleles. None of the loci were found to deviate from neutrality as revealed by F_{ST}-based outlier tests (data not shown). Deviation from HWE was found at some loci at some sampling sites; subsequent analyses with and without the allele calls for those individuals at those loci produced highly similar results (data not shown). Non-random association of genotypes was found in only eight of the 900 pairwise tests for linkage disequilibrium (Gmu21/Gmu28 in NI, RO, SI, TE and WE, Gmu21/Gmu62 in BA and BE and Gmu62/Terp2 in RO). As these patterns were not consistent across sites, all downstream analyses were based on the nine retained microsatellite loci. The final dataset included 5.7% missing data.

Across the nine microsatellite loci, there was an average of 5.2 alleles in each sampling site, with 17 private alleles detected within 10 sites (Table 2.1). Overall H_o and H_e were 0.681 and 0.681, respectively (Table 2.1).

Significant pairwise population differentiation was detected across most comparisons (Table 2.2). A weak pattern of isolation by distance was detected (r^2=0.194, P<0.001) based on Mantel tests. The genetic friction map (Figure 2.3) revealed a higher degree of genetic friction (defined here as genetic difference per unit of geographic distance) along the coast, which corresponds to sites sampled on islands and the nearby mainland.

Table 2.2 Population pairwise matrix of θ values for BC western painted turtles

	BA	BE	BL	CV	DO	EL	ER	JO	KL	LO	NI	OF	PA	RE	RV	RO	RU	SI	SC	SH	SK	SW	TE	TI
BE	0.10*																							
BL	0.05*	0.10*																						
CV	0.09*	0.06*	0.07*																					
DO	0.08*	0.10*	0.05*	0.03*																				
EL	0.09*	0.11*	0.06*	0.04*	0.01*																			
ER	0.11*	0.10*	0.09*	0.05*	0.05*	0.05*																		
JO	0.15*	0.14*	0.10*	0.07*	0.03*	0.02*	0.10*																	
KL	0.19*	0.23*	0.20*	0.18*	0.18*	0.20*	0.22*	0.24*																
LO	0.11*	0.11*	0.08*	0.03*	0.02*	0.03*	0.04*	0.07*	0.20*															
NI	0.14*	0.12*	0.11*	0.14*	0.13*	0.15*	0.20*	0.17*	0.26*	0.17*														
OF	0.04*	0.10*	0.02*	0.07*	0.07*	0.07*	0.09*	0.13*	0.17*	0.08*	0.09*													
PA	0.13*	0.18*	0.08*	0.14*	0.10*	0.13*	0.21*	0.17*	0.18*	0.17*	0.19*	0.09*												
RE	0.01*	0.09*	0.02*	0.07*	0.06*	0.08*	0.08*	0.14*	0.15*	0.08*	0.15*	0.02*	0.07*											
RV	0.03	0.09*	0.04*	0.07*	0.06*	0.08*	0.05*	0.12*	0.17*	0.09*	0.11*	0.03*	0.11*	0.03*										
RO	0.12*	0.10*	0.09*	0.05*	0.07*	0.07*	0.07*	0.12*	0.21*	0.10*	0.17*	0.09*	0.14*	0.07*	0.10*									
RU	0.13*	0.18*	0.13*	0.12*	0.10*	0.13*	0.16*	0.15*	0.04*	0.14*	0.20*	0.12*	0.12*	0.10*	0.12*	0.16*								
SI	0.15*	0.16*	0.09*	0.16*	0.15*	0.15*	0.18*	0.18*	0.29*	0.15*	0.17*	0.12*	0.22*	0.14*	0.12*	0.19*	0.24*							
SC	0.04*	0.09*	0.03*	0.09*	0.07*	0.08*	0.08*	0.13*	0.17*	0.09*	0.13*	0.03*	0.09*	0.02*	0.03*	0.10*	0.12*	0.12*						
SH	0.08*	0.10*	0.05*	0.10*	0.09*	0.08*	0.12*	0.11*	0.22*	0.11*	0.13*	0.06*	0.11*	0.03*	0.07*	0.11*	0.16*	0.09*	0.06*					
SK	0.08*	0.16*	0.08*	0.12*	0.10*	0.11*	0.17*	0.14*	0.17*	0.15*	0.15*	0.09*	0.09*	0.07*	0.09*	0.16*	0.10*	0.21*	0.10*	0.11*				
SW	0.10*	0.12	0.10*	0.15*	0.12*	0.13*	0.20	0.20*	0.34*	0.16*	0.22*	0.14*	0.20	0.12*	0.11*	0.14*	0.26*	0.23*	0.12*	0.16	0.17*			
TE	0.18*	0.22*	0.15*	0.18*	0.18*	0.19*	0.26*	0.22*	0.19*	0.21*	0.23*	0.12*	0.15*	0.12*	0.18*	0.19*	0.11*	0.28*	0.17*	0.17*	0.15*	0.32*		
TI	0.12*	0.13*	0.07*	0.06*	0.00	0.01*	0.07*	0.03*	0.22*	0.04*	0.16*	0.09*	0.15*	0.11*	0.10*	0.10*	0.13*	0.16*	0.10*	0.11*	0.12*	0.16*	0.20*	
WE	0.15*	0.22*	0.14*	0.18*	0.17*	0.18*	0.25*	0.20*	0.22*	0.19*	0.25*	0.11*	0.15*	0.17*	0.16*	0.22*	0.13*	0.27*	0.12*	0.20*	0.16*	0.25*	0.17*	0.18*

* indicates values that are statistically significant after correction for false discovery rate, $P_{citical} < 0.049$.

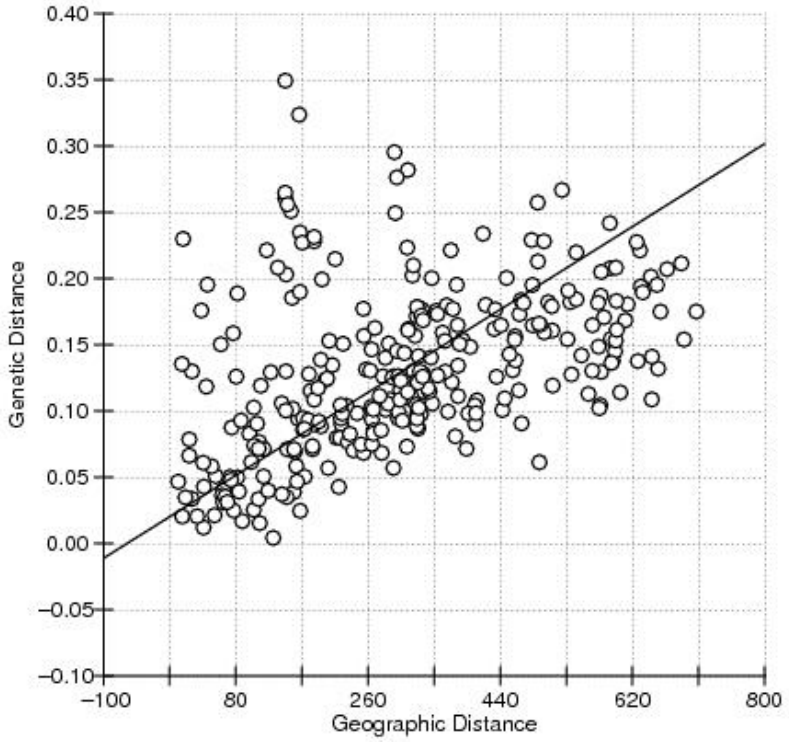


Figure 2.3 Relationship between the pairwise genetic distance (θ) and geographic distance between sites showing a weak (r^2= 0.194) but significant (P<0.001) trend of isolation by distance.

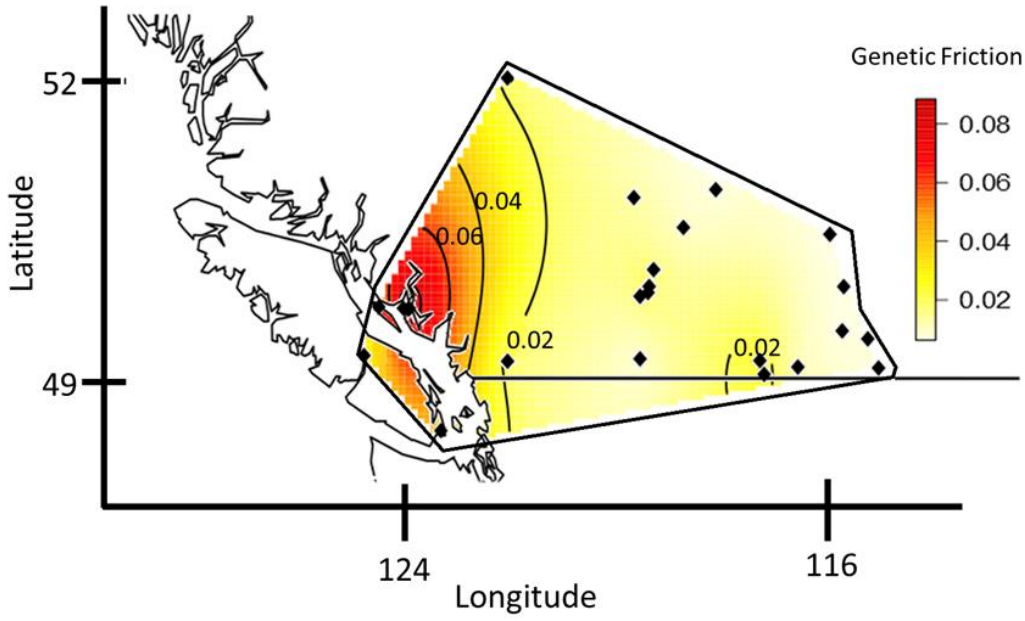


Figure 2.4 Genetic friction map computed for BC western painted turtles

The black outline delineates the sampled area, diamonds are the locations of the sampling sites. Degree of genetic friction indicated by inset legend

Migration rates, calculated as the proportion of migrants per generation, ranged from 0.01 to 0.15, with a mean of 0.01 (Table B.4). The approach implemented in BAYESASS has been deemed reliable for estimating migration rates in situations where $F_{ST} > 0.05$ and migration rates are less than $m = 0.1$ (Faubet et al. 2007). Both of these assumptions hold for the vast majority of population pairs in this study (Table 2.2, Table B.4).

STRUCTURE analyses revealed three genetic clusters in BC, with the distribution of the ad hoc statistic ΔK modal at K=3 (ΔK = 433.26; Figure 2.4a). The three clusters were Sunshine Coast-Gulf Island-Vancouver Island, South Coast-Thompson-Okanagan-Cariboo and the Kootenay (Figure 2.4a). Although three clusters were identified as the most likely, the Sunshine Coast-Gulf Island-Vancouver Island and South Coast-Thompson-Okanagan-Cariboo clusters exhibited significant heterozygote deficit potentially due to unrecognized population subdivision (Wahlund 1928). STRUCTURE was run again separately for the clusters with heterozygote deficit using the admixture model, with the ad hoc statistic ΔK modal at K=3 (ΔK = 250.17; Figure 2.4b) for the Thompson-Okanagan, Cariboo and South coast cluster, and K=2 (ΔK = 227.37; Figure 2.4b) for the Sunshine Coast-Gulf Island-Vancouver Island cluster.

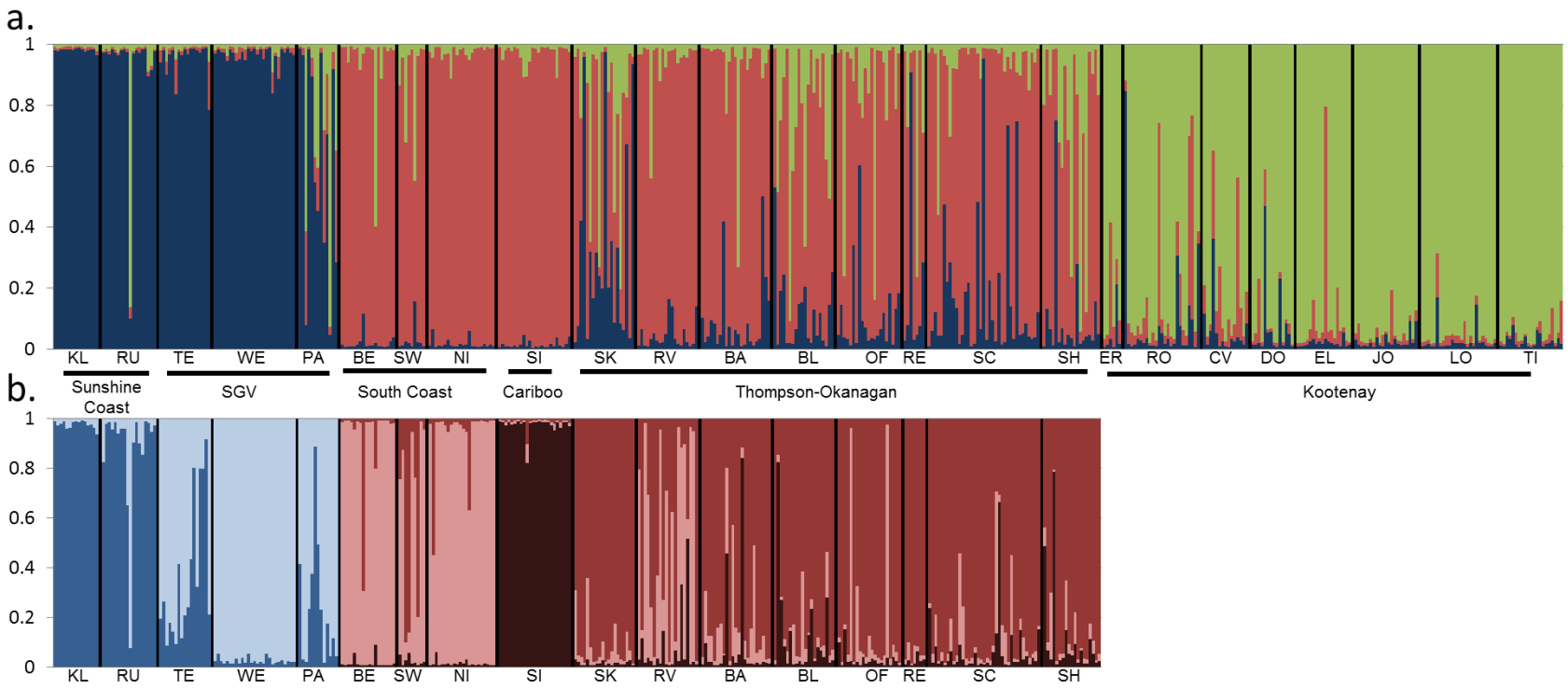


Figure 2.5 STRUCTURE bar plots using a straight admixture model for a. all populations and b. reanalysis of substructure within two broader clusters

Each colour represents an inferred genetic cluster. Each bar on the x-axis represents an individual, with the y-axis displaying the proportion of membership in each genetic cluster

Overall, the recovered clusters from the STRUCTURE analyses generally correspond to the following: Sunshine coast (KL, RU), Sunshine coast-Gulf Island-Mid-Vancouver Island (hence forth abbreviated to SGV; TE, WE, PA), South coast (BE, SW, NI), Cariboo (SI), Thompson-Okanagan (BA, BL, OF, RE, RV, SC, SH, SK) and Kootenay (ER, RO, CV, DO, EL, JO, LO, TI).

The series of AMOVA analyses revealed that among-population component of genetic variation was maximized by the six groups based on the geographically cohesive STRUCTURE clusters to a greater extent than by the 10 ecological drainage units, three faunal provinces or the current DUs (Table 2.3).

Table 2.3 Analysis of Molecular Variance (AMOVA) results showing the percentage of variation described by each hierarchical level for the following four grouping strategies: a) genetic clusters informed through the STRUCTURE analysis; b) current designatable units; c) faunal provinces; and d) ecological drainage units

	Among Groups	Among Sites within Groups	Within Sites
a. Genetic Clusters (n = 6)	7.37	5.74	86.89
b. Designatable Units (n = 2)	2.3	10.57	87.57
c. Faunal Provinces (n = 3)	3.63	9.97	86.39
d. Ecological Drainage Units (n = 10)	5.68	6.54	87.78

2.3.3 Demographic history

The estimated effective population size of each group ranged from 10.1 in the SGV to 109.7 in the Thompson-Okanagan (Table 2.4). The heterozygote excess and mode-shift tests were significant only in the Cariboo (Table 2.4). The *M*-ratios were significantly less than M_c for all values of θ (Table 2.4), indicating historical bottlenecks in all six groups. Historical

population expansion was only detected in the South coast group, and only by the *k* test (Table 2.4). The relatedness at the site level ranged from 0.02 to 0.46, with an average of 0.2 ± 0.02 SE (Table 2.1). F_{IS} ranged from -0.22 to 0.13 with an average of 0.00 ± 0.02 SE..

Table 2.4 Demographic history and effective population sizes of genetic clusters identified through the STRUCTURE analysis

	N	P_A	N_e	Wilcoxon	Mode Shift	k test P-value	g test P-value	θ	M	M_C
Sunshine Coast	33	0	34.6 (18.4 - 96.5)	ns	Normal	0.46	0.85	0.03	0.58*	0.85
								0.3	0.58*	0.82
								0.9	0.58*	0.78
SGV	57	1	10.1 (7.4 - 13.4)	ns	Normal	0.72	0.93	0.03	0.64*	0.85
								0.3	0.64*	0.82
								0.9	0.64*	0.78
South coast	49	10	11.3 (9.1 - 14.0)	ns	Normal	0.02*	0.83	0.03	0.59*	0.85
								0.3	0.59*	0.82
								0.9	0.59*	0.78
Cariboo	24	0	10.8 (6.3 - 19.2)	HE*	Shifted	0.89	1.05	0.03	0.55*	0.85
								0.3	0.55*	0.81
								0.9	0.55*	0.77
Thompson-Okanagan	167	4	109.7 (87.5 - 141.7)	ns	Normal	0.89	0.51	0.03	0.77*	0.85
								0.3	0.77*	0.82
								0.9	0.77*	0.78
Kootenay	145	11	67.5 (57.1 - 80.6)	ns	Normal	0.72	0.67	0.03	0.76*	0.85
								0.3	0.76*	0.82
								0.9	0.76*	0.78

N number of individuals, P_A number of private alleles, N_E effective population size with 95% confidence interval, *ns* not significant, *HE* heterozygote excess, M M-ratio, M_C M-critical, * indicates values are those that are statistically significant at $p = 0.05$

2.4 Discussion

2.4.1 The extent and distribution of genetic variation

My analyses show that BC populations of western painted turtle contain unique genetic diversity compared to other populations in North America, as evidenced by my findings of novel CR haplotypes not previously detected within their North American range. Fossil evidence shows that *Chrysemys* was distributed as far north and west as Nebraska during the Miocene (Holman 1976; Holman and Sullivan 1981), but there is no reported fossil evidence in BC to suggest its presence in that area prior to the Wisconsinan glaciation. Consequently, Pleistocene epoch phenomena, such as the controversial Haida Gwaii glacial refugium hypothesized to span parts of Vancouver Island, likely did not play a role in the development of regional patterns of genetic variation in BC (Demboski et al. 1999; Byun et al. 1997; Byun et al. 1999). Rather, the star-like pattern of *C. p. bellii* haplotypes revealed in the network analysis (Figure 2.2) is consistent with an interpretation of post-glacial expansion to the north and west as suggested by Starkey et al (2003), continuing into BC.

Within BC, CR haplotype 1 is common across all regions, with three other haplotypes found in one (CR haplotype 4) or two sites (CR haplotypes 2, 3), respectively (Figure 2.2, Table B.1). This lack of regional structuring of mtDNA variation is in contrast to the signal detected from the microsatellite genotypic data. The six discrete groups delineated through the STRUCTURE analysis have distinct patterns of genetic variation, although in some instances the driving factor behind those regional patterns is unclear. Anthropogenic movement of turtles is common in modern times (E. Jensen, personal observation), and it is possible that translocation also occurred

historically. Such an occurrence may explain the genetic similarity among turtles from Texada Island and Port Alberni, and the lower mainland and southern Vancouver Island, which are geographically separated by deep ocean in both instances. However, there is no genetic evidence for a founder event at any of those sites based on heterozygote excess and mode-shift tests (data not shown), and natural colonization cannot be ruled out.

Pairwise comparisons found significant differences in microsatellite allele frequency distributions at the site level, with a weak trend of differentiation increasing with geographic distance between sites. However, the non-stationary genetic friction map (Figure 2.3) revealed a greater amount of genetic difference per unit geographic distance along the coast relative to other sampled areas. These trends were also apparent within my estimates of gene flow, with migration rates between most water bodies estimated at less than 0.1, which is the threshold for being considered demographically independent (Hastings 1993). The three comparisons that exhibited migration rates greater than 0.1 indicated unidirectional migration between populations that are still significantly differentiated (EL to JO, TI to JO, KL to RU), although θ values are low (Tables 2.2 and Table B.4).

Despite each site being genetically differentiated, the levels of within-site genetic diversity, as represented by H_e (ranging from 0.51 to 0.77), are comparable to the range reported in other species of freshwater turtle (*Emydoidea blandingii* 0.45 to 0.71, Mockford et al. 2007; *Glyptemys muhlenbergii* 0.57 to 0.70, Pittman et al. 2011). Although some of the sites have high mean pairwise relatedness (KL, NI, PA, RU, SI, SW, TE, WE), in most instances this is not accompanied by high levels of inbreeding, which was detected at only two sites (BL and TE;

Table 2.1). These results suggest that local population sizes are still sufficiently large to counteract the loss of variation due to drift.

2.4.2 Demographic history of BC western painted turtles

The range of the western painted turtle likely only extended into BC after ~12,000 years ago due to the Wisconsin glaciation, and potentially much later if modern expansion west of Chicago was not possible prior to 14,000 years ago, as suggested by Starkey et al. (2003). Here, we only detected a genetic signature of historical population expansion in one group (south coast, Table 2.4). Although this study has the appropriate sample size for *k* and *g* tests, it is recommended that at least 25 microsatellite loci be used, and that the mutation rates of loci are uniform. My panel of nine microsatellites included both di- and tetra-nucleotide repeat loci, which are known to have variable mutation rates (Chakraborty et al. 1997). Moreover, methods for detecting population expansion, including the *k* and *g* tests employed here as well as others (e.g. the imbalance index, Kimmel et al. 1998), have low statistical power when detecting events that occurred fewer than 1000 generations ago (King et al. 2000). The generation time of *Chrysemys* has not been calculated, but the IUCN listing suggests approximately 20 years (Van Dijk 2011). Given this estimate, post-glacial expansion occurred less than 700 generations ago and current methods are limited in their ability to detect that event. Consequently, the results of the *k* and *g* tests reported here should be interpreted with caution. Expansion into the north and western limit of the species' range were likely accompanied by founder effects, detected in this study in all six groups as historical bottlenecks indicated by the *M*-ratio tests.

2.4.3 Conservation unit concepts

Overall, my results reflect the difficulties of designating units below the species level. The presence of novel mtDNA haplotypes suggest that BC contains unique genetic variation that may be important to conserve. However, it is not clear how this information can be used to prioritize populations within BC for conservation. For example, following the ESU definition based on reciprocal monophyly (Moritz 1994), all BC western painted turtles would be lumped into a single ESU. The major CR haplotype (number 2) is found across the province, including at sites containing other haplotypes (1, 3 and 4), thus not meeting the criterion of reciprocal monophyly. At a finer-level, defining a management unit as a group of populations that show significant divergence of allele frequencies at nuclear or mitochondrial loci (Moritz 1994) would result in each occupied site as a separate MU for western painted turtles (Table 2.2). From a management perspective, it is likely not practical to devise conservation strategies at the site-level on a province-wide scale.

The more flexible DU concept provides alternative units for this species. Based on the dual criteria of genetic distinctiveness (via STRUCTURE) and range disjunction, there are six discrete units of western painted turtle in BC: Sunshine coast, SGV, south coast, Cariboo, Thompson-Okanagan, and Kootenay. Assessing the significance of these units is more difficult, as the guidelines are largely value-based to allow policy makers the flexibility to decide which kind of intraspecific variation to recognize. The wording of the criteria leaves them poorly defined and without quantifiable measures. For example, one criterion for recognition is “loss of the unit would result in an extensive gap of the range of the species in Canada” (COSEWIC 2008). If the proposed Thompson-Okanagan unit of western painted turtles were to disappear,

the Kootenay and Cariboo units would be even more disjunct from one another, as would the Sunshine coast and Thompson-Okanagan if the south coast unit were to disappear. As there is little guidance on the scale required for an "extensive gap", the significance of each unit based on this criterion is ambiguous. Similarly, although the Cariboo unit is potentially the most northern locality for this wide-ranging species, it is unclear whether this circumstance constitutes "a unique ecological setting", another significance criterion by which DUs may be recognized.

Criteria for defining ESUs are by no means universally agreed upon, with some authors arguing that no single concept will be appropriate for all taxa (Fraser and Bernatchez 2001). Because turtle mitochondrial DNA evolves eight times slower than that of other vertebrates (Avise et al. 1992), Moritz's condition of reciprocal monophyly of mitochondrial DNA haplotypes to define evolutionarily distinct lineages may not be suitable. Turtle populations may evolve along independent trajectories for hundreds of generations, spanning many thousand years before this criterion is met, long after demographic independence and separate management for conservation purposes is warranted. Moritz (2002) acknowledges that the evolutionary processes within a taxon should be considered and strategies based on relevant criteria should be applied, however, no guidance is given on how to incorporate this within the ESU framework.

The guidelines for defining DUs as implemented by COSEWIC are perhaps more robust to considering various organisms with widely different evolutionary histories and ecologies. The analyses here show that dividing the range of the western painted turtle in BC using the generic faunal provinces as the basis for delineation of the Pacific Coastal and Intermountain-Rocky Mountain DUs does a poor job of describing genetic variation, with almost four times as much

variation partitioned within units than between them (Table 2.3). In contrast, the ecological drainage units, which are based on the interaction between the distribution of freshwater fish species, and the physical characteristics and environmental processes that characterize the ecosystem (Ciruna et al. 2007), did represent the distribution of genetic variation within the province reasonably well. However, this may not be the case for other organisms and using genetic methods can be a robust and effective way to determine units that are both biologically meaningful and practical in scale for conservation purposes. Nevertheless, if a species specific assessment is not possible, the ecological drainage units may represent a better biogeographic grouping strategy than the faunal provinces for aquatic organisms in BC.

As the ultimate goal of identifying conservation units is to preserve genetic variation to allow future adaptation, some authors have argued that adaptive divergence should be used to inform such designations (e.g. Kohn et al. 2006; Ouborg et al. 2009; Reed and Frankham 2001; Sgro et al. 2011; Bonin et al. 2007; Funk et al. 2012). Despite this increasing awareness and the technological advances that allow for the detection and assaying of adaptive genetic variation within natural populations, there are still no generally accepted methods for incorporating this information into conservation prioritization (but see Bonin et al. 2007; Funk et al. 2012). Although beyond the scope of the current study, future research targeting adaptive genetic variation could help resolve uncertainties about the significance of the six discrete units proposed here for western painted turtles, especially given the large ecological variation observed across BC.

2.4.4 Management implications

My results demonstrate that there are six discrete units of western painted turtles in BC, corresponding to the Sunshine coast, SGV, south coast, Cariboo, Thompson-Okanagan and Kootenay (Figure 2.1). I suggest that these six units require further consideration to determine whether they meet the significance criteria to become DUs. Three of these units (SGV, south coast and Cariboo) are characterized by very small effective population sizes N_e (<15), evidence of significant historical bottlenecks, and embedded sites exhibiting signatures of recent bottlenecks (data not shown) indicating that these units warrant independent management. Across the province, each occupied water body is demographically independent and qualifies as a MU under Moritz (1994), suggesting that demographic and population monitoring should occur at the site level, especially in the small, isolated populations in SGV and the Cariboo.

Overall, the situation of the western painted turtle in BC clearly illustrates the difficulties of designating units below the species level. It is imperative, however, that units are delineated that will recognize and protect the novel genetic diversity detected at their northern range margin in BC. The main threats to BC western painted turtles cited in the 2006 COSEWIC status assessment were road mortality and habitat loss. These pressures have not diminished since its publication. Although road density is much higher in the other units, in the Cariboo at least 12 adult individuals were killed by vehicles on a single 200 meter stretch of highway in the summer of 2012 alone (unpublished data). Moreover, high human population density and associated development in the south coast unit will continue to negatively impact the quality of remaining turtle habitat in that area. Since historical times, 85% of wetlands in the south Okanagan and the Fraser River delta have been converted into agricultural lands (Boyle et al. 1997; B.C. Ministry

of Environment Lands and Parks 1993) and the remaining remnants are still under threat. Quality habitat and viable population sizes are needed for the western painted turtle to endure in BC. Based on my findings, the conservation value of each of the six discrete units should be recognized and management plans designed to maximize population persistence throughout the province.

3 Chapter: INVESTIGATING TAXONOMIC UNCERTAINTY IN PAINTED TURTLES

3.1 Background

Species are the fundamental units of biodiversity, and are an important concept in how humans view nature (Richards 2010). Despite the importance of species, there is ongoing controversy over species concepts and definitions, which has major implications for the number and composition of described species, which in turn affects how we understand ecological and evolutionary processes. Determining where the so-called "species boundary" lies and which groups of organisms are above it (separate species) or below it (lineages within the same species) is particularly difficult in recently diverged lineages (Shaffer and Thomson 2007). There are both theoretical challenges associated with deciding how much divergence is necessary for taxonomic distinction to be appropriate and practical challenges for disentangling relationships using genetic or morphological evidence.

A commonality among many species concepts is that species should be separately evolving metapopulation lineages (De Queiroz 2007); if one accepts this, then the challenge is to determine how to identify the boundaries between such lineages. The methodologies of phylogenetics provide a whole toolbox for determining cladogenetic relationships; however, such methods can only be legitimately applied to organisms that are in fact related by nested, hierarchical relationships (Goldstein et al. 2000). If organisms are instead related through patterns of ancestry and descent (i.e. reticulating genealogical relationships), phylogenetic analysis can impose hierarchical structure, thus producing spurious results (Goldstein et al.

2000). In such cases, network-based analyses which do not impose the criterion of bifurcation can be a powerful tool for visualizing relationships (Posada and Crandall 2001).

Concomitant with the use of an appropriate analytical framework, the choice of molecular marker is critical for accurately reconstructing evolutionary history among recently diverged taxa. Both mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) can be used for reconstructing evolutionary history and each has specific characteristics that can be used to approach different points along a divergence continuum. MtDNA evolves more rapidly than nuDNA, given its lower effective population size (¼ of nuDNA) and resulting accelerated rate of lineage sorting (Funk and Omland 2003). Moreover, mtDNA is maternally inherited, precluding significant levels of recombination characteristic of nuDNA. However, these attractive properties that make mtDNA useful for reconstructing recent events also contribute to higher rates of homoplasy or convergent mutations that can potentially obscure the signal of more distant events (Zink and Barrowclough 2008). Because it is uniparentally inherited, mtDNA can only trace the lineage of one of the sexes, which may be a biased portrayal of history if, for example, there was sex biased dispersal. The differences between mtDNA and nuDNA can result in the reconstruction of evolutionary history that is discordant across markers.

Wide ranging species that occupy diverse ecosystems can also be a challenge for taxonomy, as they may contain several lineages at various stages of divergence from one another. Across the species' range, populations can experience disparate selection pressures resulting in local adaptation (Gaston 2003). Peripheral populations may also experience limited gene flow with the core of the range, which can result in divergence and ultimately peripartic speciation (Lesica and

Allendorf 1995). Recent genetic studies of such taxa have resulted in various outcomes from the description of several species with limited distributions (e.g. Rodriguez-Robles and De Jesus-Escobar 2000) to the finding of continuously shared gene pools across the ranges of several subspecies (e.g Burbrink et al. 2000).

Chrysemys (painted turtles, Schneider 1783), a genus within the family Emydidae, is a wide ranging group experiencing ongoing taxonomic debate with regards to the status of the four formally described morphological types. Currently, the Society for the Study of Amphibians and Reptiles recognizes two species: *Chrysemys dorsalis* and *Chrysemys picta*, the latter of which is composed of three subspecies: *C. p. picta, C. p. bellii* and *C. p. marginata* (Crother 2012). These designations are largely based on a phylogenetic study that used partial sequences of the mitochondrial control region (CR) of individuals across the United States, and showed *C. dorsalis* as a divergent evolutionary lineage from the other three morphotypes (Starkey et al. 2003). Other taxonomists disagree with the elevation of *dorsalis* to the species level, and argue that it does not have either geographic or reproductive isolation from the other morphotypes within *Chrysemys* (Ernst and Lovich 2009). Interbreeding and hybridization are common wherever the ranges of the morphotypes overlap (Weller et al. 2010) to the extent that some speculate that a continuously shared gene pool may exist over the entire range of *Chrysemys* (Ernst and Lovich 2009). For this reason, many taxonomists only tentatively accept *C. dorsalis* as a species and often still refer to *Chrysemys* as a monotypic genus with four subspecies (Fritz and Havas 2007). However, Starkey et al. (2003) did not find *picta, marginata* and *bellii* to be separate evolutionary lineages, and suggested that their subspecific taxonomic descriptions be dropped. Although there are morphological differences between *picta, marginata* and *bellii*,

those differences are based on morphometric averages that do not provide clear distinctions and cannot be used to diagnose individuals (Ultsch et al. 2001).

Here, I supplement the study of Starkey et al. (2003) with overlapping mtDNA CR sequence data from the northern range margin of painted turtles in Canada, as well as new range-wide sequence data from the PAX-P1 nuclear intron to further investigate taxonomic uncertainty and palaeogeography within the group. Moreover, I conducted comparative network and phylogenetic analyses to examine these different approaches and to explore the implications for providing insights at the species boundary.

3.2 Methods

3.2.1 Sampling

Blood samples were collected from painted turtles across British Columbia, as described in Chapter 2. Previously collected painted turtle tissue samples from across the USA and Ontario were obtained from the archives of Dr. Bradley Shaffer (University of California, Los Angeles) and the Queen's University Biological Station, respectively. Due to the limitations of using archival samples, the number of samples of each morphotype available varied.

3.2.2 Data collection

A 665 base pair segment of the mitochondrial genome, including part of the control region (CR) was amplified as described in Chapter 2. A 644 base pair segment of the PAX-P1 intron was amplified as a single fragment using PAX.20F and PAX.21R primers (Kimball et al. 2009). PCR was carried out on an ABI Veriti thermal cycler in 25 µl reactions containing ~20-40 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 7.5µg bovine serum albumin, 0.4µM each primer and 0.5 AmpliTaq Gold DNA polymerase (AB Biosystems). Cycling conditions consisted of 95˚C for 10 minutes, 35 cycles of 95 ˚C for 30 seconds, 55 ˚C for 1.15 minutes, 72 ˚C for 1.5 minutes, and a final extension at 72 ˚C for 10 minutes.

The sequencing reactions were performed using ABI BigDye v3.1 Terminator chemistry and sequences were run on an Applied Biosystems 3130XL DNA automated sequencer. Sequences were visualized and edited using Sequencher 5.0 (Gene Codes Corporation).

In addition to the CR and PAX-P1 sequence data collected here, exemplar sequences from across the range of the painted turtle were downloaded from GenBank and contributed by collaborators (see Appendix C, Table C.1). Outgroup taxa consisted of exemplar individuals of *Platysternon megacephalum, Terrapene carolina, Emys marmorata, Pseudemys texana, Graptemys geographica* and *Trachemys scripta elegans* (Table C.2). For all tree-based analyses *Platysternon megacephalum* was selected as the outgroup as it is sister to the family Emydidae (Guillon et al. 2012).

3.2.3 Population genetic analysis

The CR and PAX-P1 data sets were grouped into individuals from localities within the range of each of the four morphotypes; individuals potentially from hybrid zones were excluded. The resulting data set for the CR included 151 *bellii*, 49 *marginata*, 40 *picta* and 13 *dorsalis* sequences (Table C.1, Figure 3.1) and the data set for PAX-P1 included 77 *bellii*, 17 *marginata*, 9 *picta* and 4 *dorsalis* sequences (Table C.1, Figure 3.1).The sequences for CR and PAX-P1 were aligned separately in Geneious (Biomatters Ltd.) using MUSCLE (Edgar 2004) and default settings. A microsatellite and adjacent flanking region within the PAX-P1 contributed to alignment ambiguity and were subsequently removed.

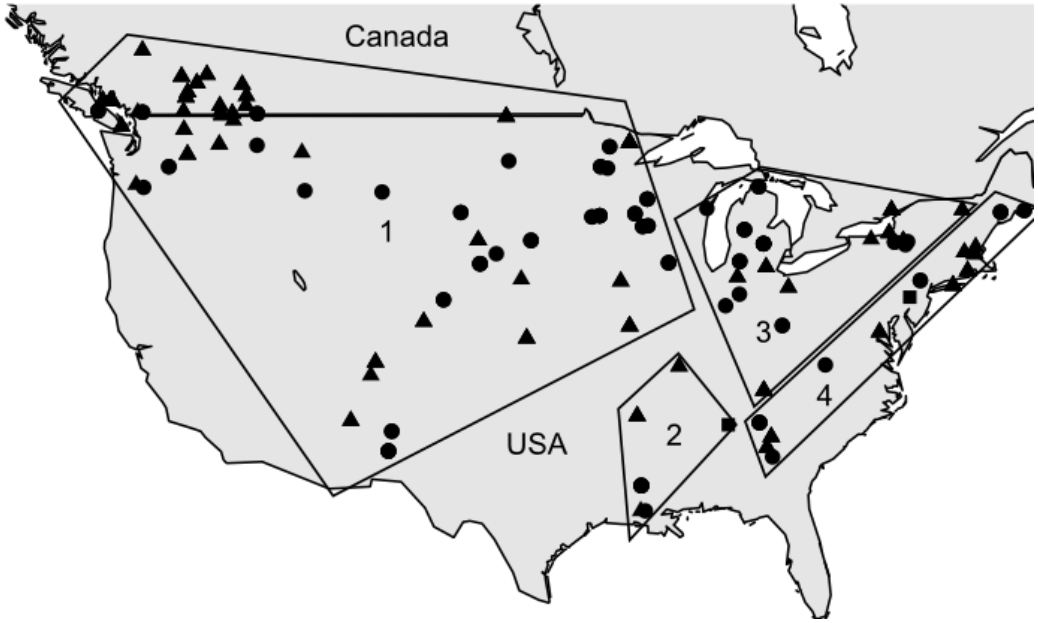


Figure 3.1 Localities for *Chrysemys* individuals used in the population genetic analyses, including those based on both the mitochondrial control region (CR) and PAX-P1 intron (triangles), CR only (circles and triangles) and PAX-P1 intron only (triangles and squares).

Polygon numbers indicate samples falling within the described distributions of *bellii* (1), *dorsalis* (2), *marginata* (3), and *picta* (4).

From the nuclear PAX-P1 sequences, haplotypes were reconstructed using the Bayesian inference method implemented in PHASE 2.1 (Stephens and Donnelly 2003; Stephens et al. 2001) using an input file generated by SeqPHASE (Flot 2010). PHASE was run five times using a run length of 100 iterations with a burn-in of 100, with a relaxed assumption of the stepwise mutation model. The haplotypes reconstructed from the run with the best average goodness-of-fit were used in further analyses.

Haplotypic and nucleotide diversity estimates for each morphotype were calculated from the CR and PAX-P1 haplotypes separately in ARLEQUIN (Excoffier et al. 2005). For each gene region, levels of genetic divergence among samples were calculated with the fixation index, F_{ST}, using the Tamura Nei model (Tamura and Nei 1993), as implemented in ARLEQUIN (Excoffier et al. 2005). Significance for the pairwise F_{ST} comparisons was assessed using 3000 permutations. Genetic structure among the four morphotypes was examined using analysis of molecular variance (AMOVA) using the Tamura Nei model and 3000 permutations (Tamura and Nei 1993) as implemented in ARLEQUIN (Excoffier et al. 2005). Sequence alignments for the CR and PAX-P1 were used to identify characters that were fixed within and differing among morphotypes using population aggregation analysis (Davis and Nixon 1992).

3.2.4 Network and phylogenetic analysis

Haplotype networks were constructed for the CR and PAX-P1 haplotype data sets using statistical parsimony as implemented in TCS (Clement et al. 2000). Best-fit models of nucleotide substitution were selected for each gene region using AIC criteria in MrModeltest2 (Nylander

2004). The HKY (Hasegawa et al. 1985) model with a proportion of the sites being invariable (I) with rate variation (G) was selected for both the CR and PAX-P1.

CR haplotype trees were reconstructed based on all available sequences including those from Starkey et al. (2003) as well as the new data collected here (total n=253). The resulting operational taxonomic units (OTUs) represent all haplotypes sampled within each morphotype (n=51). A gene tree was estimated using the Bayesian framework implemented in MrBayes (Ronquist and Huelsenbeck 2003) using the HKY+I+G model. MrBayes was run with four chains for 1,100,000 generations, sampling every 200 generations with *P. megacephalum* as the outgroup. The first 25% of the trees were discarded and a consensus tree with posterior probability support threshold of 25% was produced. A neighbour joining tree was created in PAUP* (Swofford 2003) using the HKY+I+G and 100 bootstrap replicates with *P. megacephalum* as the monophyletic outgroup.

Using a subset of individuals (n=76) that covered a representative sample of the range of each morphotype, a PAX-P1 gene tree was reconstructed using the Bayesian framework implemented in MrBayes and the same parameters as above. The CR and PAX-P1 sequences were concatenated for individuals with both sequences available (n=76) and a combined phylogeny was reconstructed in MrBayes using the HKY+I+G model and the same parameters as above.

3.3 Results

3.3.1 Within and among subspecies variation

A total of 47 CR haplotypes were recovered among the 253 individuals from across the range of the four morphotypes of *Chrysemys*. The number of CR haplotypes within a morphotype ranged from six (*dorsalis*) to 18 (*marginata*), with levels of haplotypic and nucleotide diversity ranging from 0.69 to 0.84 and 0.002 to 0.007, respectively (Table 3.1). Four CR haplotypes were shared among groups (two *bellii* and *marginata*, two *marginata* and *picta*), although no haplotypes were shared by more than two morphotypes. A total of 29 PAX-P1 haplotypes were recovered among the 107 *Chrysemys* individuals. The number of PAX-P1 haplotypes within a morphotype ranged from five (*dorsalis*) to 12 (*bellii*) (Table 3.1). Eight PAX-P1 haplotypes were shared among morphotypes. Haplotypic diversity ranged from 0.65 to 0.90 and nucleotide diversity ranged from 0.002 to 0.008 (Table 3.1).

Table 3.1 Diversity indices from the analysis of the CR and PAX-P1

		N	# of Haplotypes	Haplotypic Diversity	Nucleotide diversity
CR	*bellii*	151	17	0.69 ± 0.03	0.002 ± 0.001
	marginata	49	19	0.84 ± 0.04	0.007 ± 0.004
	picta	40	9	0.77 ± 0.05	0.004 ± 0.002
	dorsalis	13	6	0.72 ± 0.13	0.002 ± 0.002
PAX-P1	*bellii*	77	12	0.65 ± 0.04	0.004 ± 0.002
	marginata	17	11	0.93 ± 0.02	0.007 ± 0.004
	picta	9	9	0.90 ± 0.04	0.008 ± 0.005
	dorsalis	4	5	0.72 ± 0.13	0.002 ± 0.002

Based on the CR, significantly greater amounts of genetic variation were structured among rather than within the samples of the four groups ($p<0.0001$, Table 3.2). A lower but still significant amount of variation was also detected among groups based on the PAX-P1 data ($p<0.0001$, Table 3.2). For both gene regions, when *dorsalis* was removed from the AMOVAs, the results were consistent (Table 3.2).

Table 3.2. Percentages of genetic variation in the CR and PAX-P1 intron that are partitioned among and within the morphotype groups determined through AMOVA

		Among Populations	Within Populations	Significance
CR	With all 4	69.69	30.31	0.0000
	dorsalis Removed	62.46	37.54	0.0000
PAX-P1	With all 4	27.15	72.85	0.0000
	dorsalis Removed	26.56	73.44	0.0000

The CR fixation indices were significant for all pairwise comparisons, with F_{ST} being greatest between *bellii* and *dorsalis* (0.90) and least between *picta* and *marginata* (0.34) (Table 3.3). Fixation indices based on the PAX-P1 haplotypes were again greatest between *bellii* and *dorsalis* (0.41) and least between *picta* and *marginata* (0.05) (Table 3.3); the only non-significant comparison was between *picta* and *dorsalis* (F_{ST}= 0.08, p=0.11).

Table 3.3 Pairwise comparisons of the fixation index, F_{ST}, based on the PAX-P1 intron above the diagonal and CR below the diagonal

	bellii	***marginata***	***picta***	***dorsalis***
bellii	-	0.3**	0.27**	0.41**
marginata	0.63**	-	0.05*	0.1*
picta	0.73**	0.34**	-	0.08
dorsalis	0.9**	0.71**	0.82**	-

* $P<0.05$, ** $P<0.001$

Across the 665 base pairs of CR sequence, five characters were fixed and different between *dorsalis* and the other three morphotypes; there are a total of five fixed differences between *dorsalis* and *marginata*, eight between *dorsalis* and *bellii* and ten between *dorsalis* and *picta*. Only one fixed difference in the CR was found between *bellii* and *picta*, and none between *bellii* and *marginata*, or *marginata* and *picta*. In the PAX-P1 intron, no fixed character differences were found among morphotypes.

3.3.2 Network and phylogenetic analysis

The haplotype network recreated based on the CR shows *dorsalis* and *bellii* to be relatively distinct from the other morphotypes, with *marginata* not appearing as a cohesive entity and largely mixed with *picta* (Figure 3.2). The main feature of the PAX-P1 network is overarching patterns of reticulation, with none of the morphotypes separating from the others (Figure 3.3).

The Bayesian gene tree for the CR (Figure 3.4A) shows *Chrysemys* to be monophyletic; *dorsalis* forms a monophyletic clade with high support (posterior probability 98), that is nested within the greater *Chrysemys* clade. The neighbour joining gene tree for the CR (Figure 3.4B) shows *Chrysemys* to be monophyletic, with *dorsalis* forming a basal, monophyletic clade similar to the pattern reconstructed in Starkey et al. (2003).

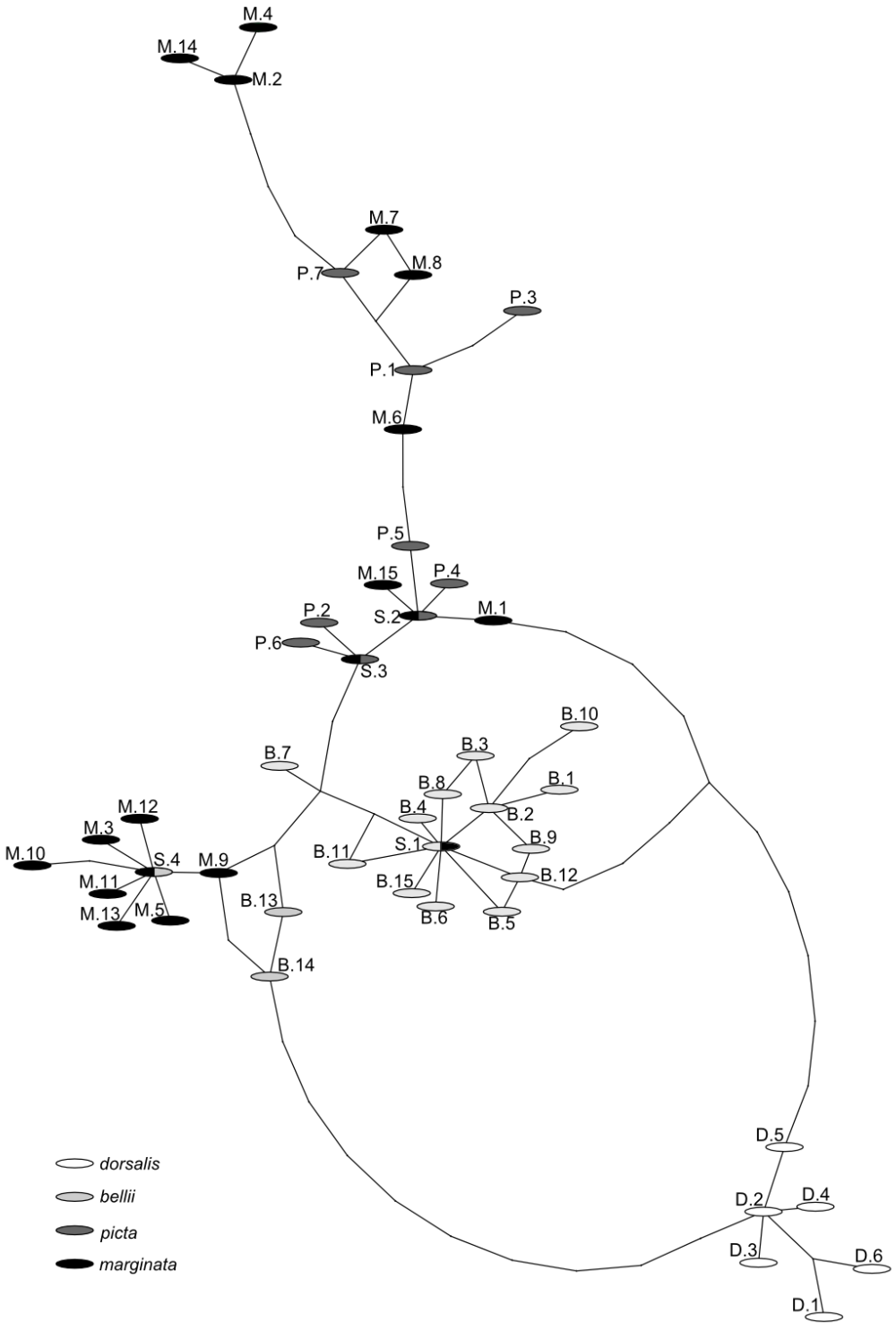


Figure 3.2 Haplotype network for the CR

Ovals indicate haplotypes in the sample, single colour ovals are those found in only one morphotype, dual coloured nodes are those detected in more than one morphotype. Frequency of the shared haplotypes are not indicated. Inferred but unsampled haplotypes are indicated as links in the network.

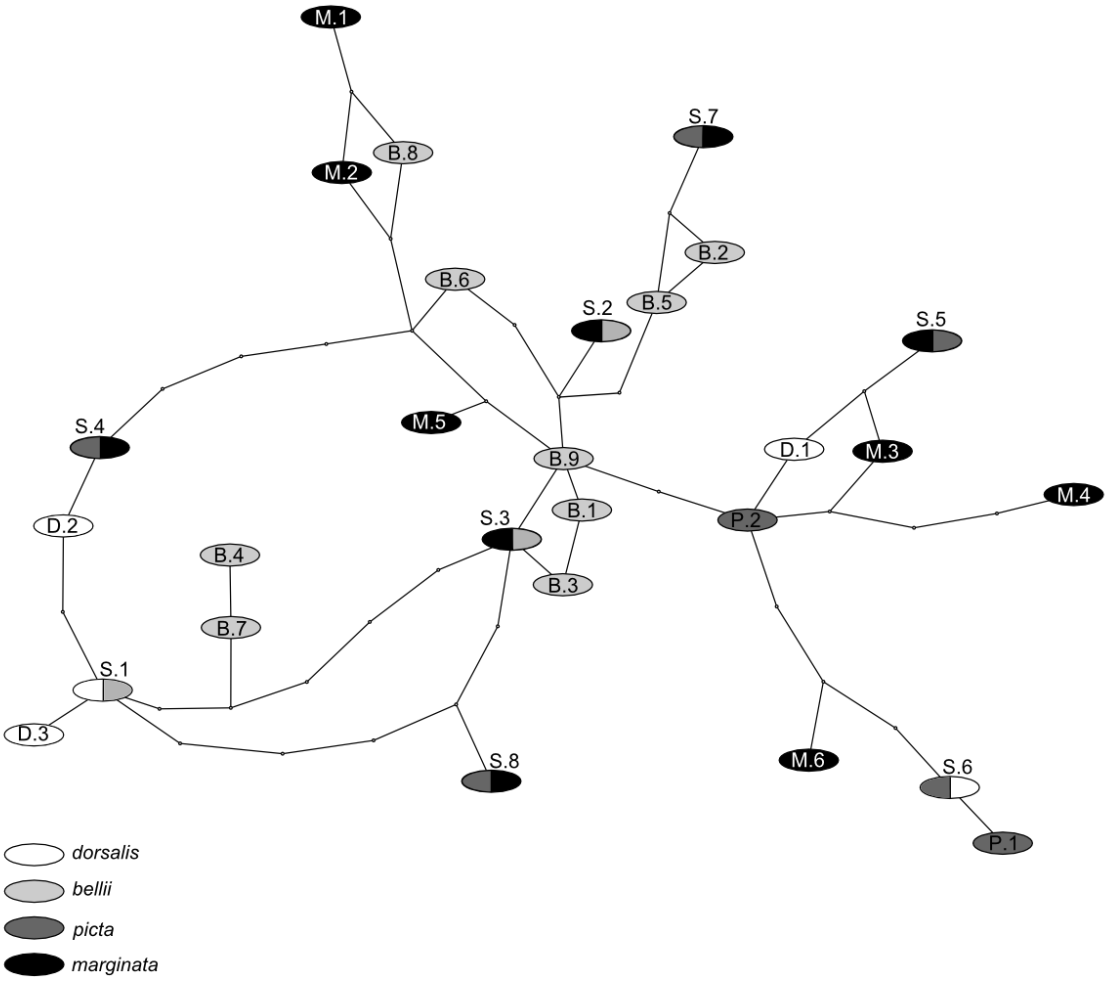
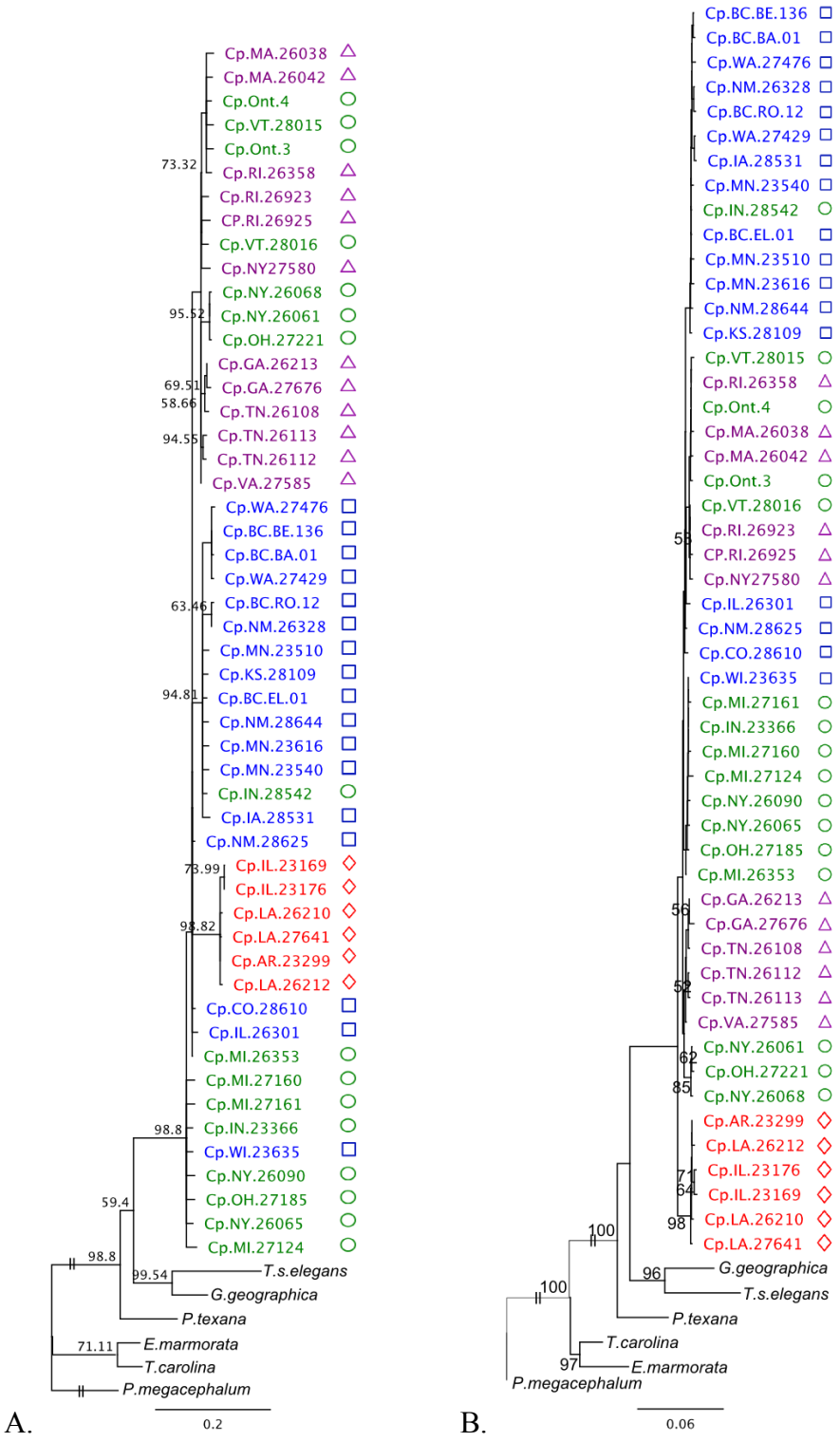


Figure 3.3 Haplotype network for the PAX-P1 intron

Ovals indicate haplotypes in the sample, single colour ovals are those found in only one morphotype, dual coloured nodes are those detected in more than one morphotype. Frequency of the shared haplotypes are not indicated. Inferred but unsampled haplotypes are indicated as links in the network.

The Bayesian gene tree analysis for PAX-P1 (Figure 3.4C) returned a poorly resolved tree in which *Chrysemys* is not monophyletic, with no support for all but the most fine-scale clustering. The Bayesian tree based on the concatenated PAX-P1 and CR sequences (Figure 3.4D) shows *Chrysemys* to be monophyletic with high support for a basal clade of western New York and Ohio *marginata*. The concatenated tree places a monophyletic *dorsalis* within the greater *Chrysemys*, with *picta* and *marginata* individuals as sister taxa with low support.


Cp.MA.26038
Cp.MA.26042
Cp.Ont.4
Cp.VT.28015
Cp.Ont.3
Cp.RI.26358
Cp.RI.26923
CP.RI.26925
Cp.VT.28016
Cp.NY27580
Cp.NY.26068
Cp.NY.26061
Cp.OH.27221
Cp.GA.26213
Cp.GA.27676
Cp.TN.26108
Cp.TN.26113
Cp.TN.26112
Cp.VA.27585
Cp.WA.27476
Cp.BC.BE.136
Cp.BC.BA.01
Cp.WA.27429
Cp.BC.RO.12
Cp.NM.26328
Cp.MN.23510
Cp.KS.28109
Cp.BC.EL.01
Cp.NM.28644
Cp.MN.23616
Cp.MN.23540
Cp.IN.28542
Cp.IA.28531
Cp.NM.28625
Cp.IL.23169
Cp.IL.23176
Cp.LA.26210
Cp.LA.27641
Cp.AR.23299
Cp.LA.26212
Cp.CO.28610
Cp.IL.26301
Cp.MI.26353
Cp.MI.27160
Cp.MI.27161
Cp.IN.23366
Cp.WI.23635
Cp.NY.26090
Cp.OH.27185
Cp.NY.26065
Cp.MI.27124
T.s.elegans
G.geographica
P.texana
E.marmorata
T.carolina
P.megacephalum
0.2
A.
Cp.BC.BE.136
Cp.BC.BA.01
Cp.WA.27476
Cp.NM.26328
Cp.BC.RO.12
Cp.WA.27429
Cp.IA.28531
Cp.MN.23540
Cp.IN.28542
Cp.BC.EL.01
Cp.MN.23510
Cp.MN.23616
Cp.NM.28644
Cp.KS.28109
Cp.VT.28015
Cp.RI.26358
Cp.Ont.4
Cp.MA.26038
Cp.MA.26042
Cp.Ont.3
Cp.VT.28016
Cp.RI.26923
CP.RI.26925
Cp.NY27580
Cp.IL.26301
Cp.NM.28625
Cp.CO.28610
Cp.WI.23635
Cp.MI.27161
Cp.IN.23366
Cp.MI.27160
Cp.MI.27124
Cp.NY.26090
Cp.NY.26065
Cp.OH.27185
Cp.MI.26353
Cp.GA.26213
Cp.GA.27676
Cp.TN.26108
Cp.TN.26112
Cp.TN.26113
Cp.VA.27585
Cp.NY.26061
Cp.OH.27221
Cp.NY.26068
Cp.AR.23299
Cp.LA.26212
Cp.IL.23176
Cp.IL.23169
Cp.LA.26210
Cp.LA.27641
G.geographica
T.s.elegans
P.texana
T.carolina
E.marmorata
P.megacephalum
0.06
B.

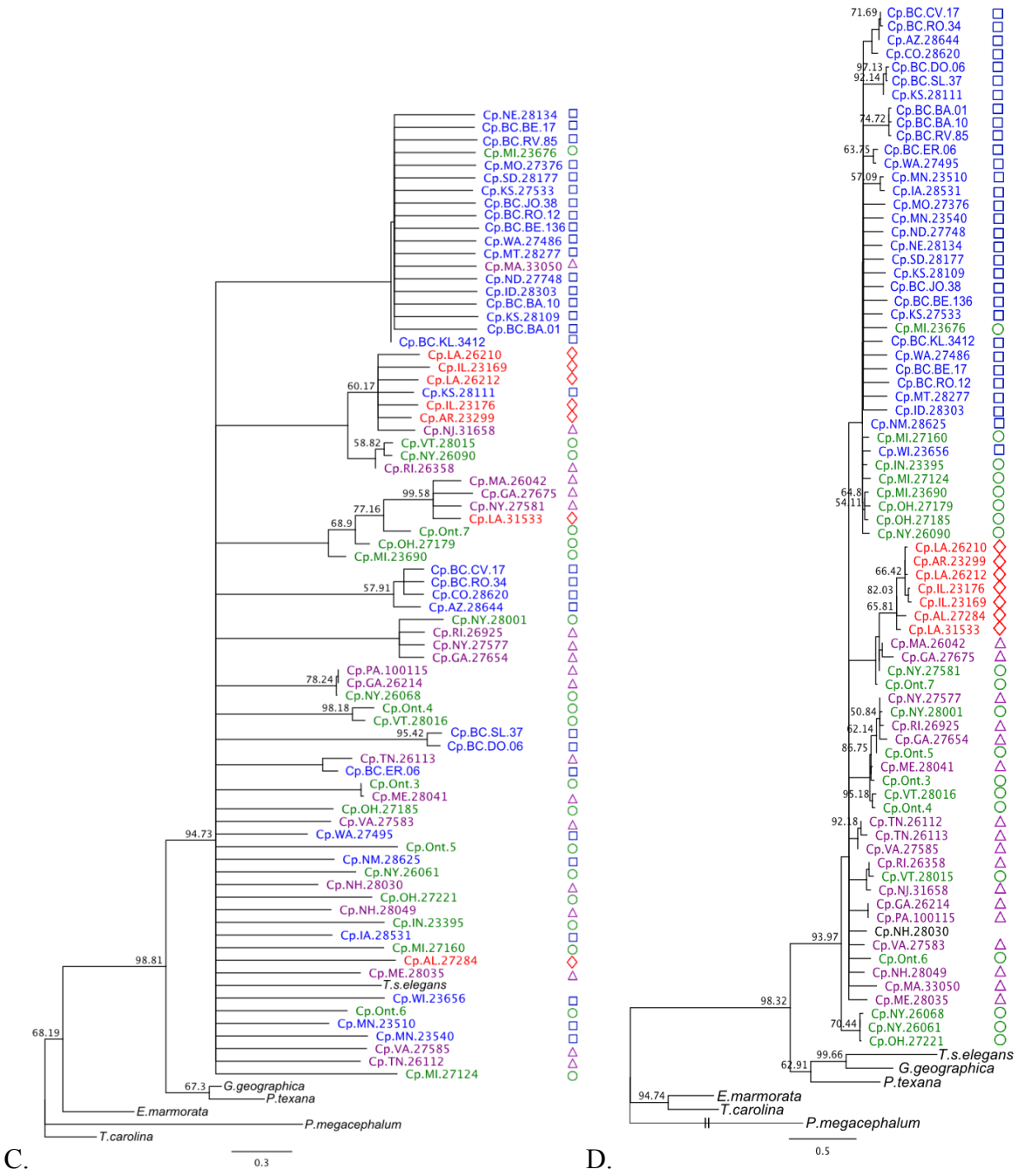


Figure 3.4 Phylogenetic trees for A. the Bayesian analysis of the CR, B. Neighbour Joining of the CR with bootstrap support indicated, C. the Bayesian analysis of the PAX-P1 intron and D. the Bayesian analysis of the concatenated sequences

Bayesian trees show clades with posterior probabilities greater than 25% but only values greater than 50% are indicated. Blue square is *bellii*, red diamond is *dorsalis*, green circle is *marginata* and purple triangle is *picta*.

3.4 Discussion

3.4.1 Uncertain taxonomy

Starkey et al. (2003) tentatively proposed that *Chrysemys* should be split into two species, *Chrysemys picta* and *Chrysemys dorsalis*, pending future analysis of nuclear genes. Here, I present data from a nuclear gene, as well as increased geographic sampling expanding into Canada, with the goal of shedding further light on the taxonomy of this group. The resolution of *Chrysemys'* taxonomy is complicated by several factors. There are four morphotypes that have been described, however, even within the core of their ranges, the morphotypes are poorly differentiated based on morphometric characters (Ultsch et al. 2001). The ranges of the morphotypes naturally overlap to form hybrid zones (Mann 2007; Ultsch et al. 2001; Weller et al. 2010). Although there is environmental variation across the range of *Chrysemys*, each morphotype spans a large distance north-south, and clinal adaptations to environmental variation would be captured within each morphotype, likely not partitioned among them. The exception to this pattern is *dorsalis*, which has a distribution that is limited to the extreme southeast of the range of *Chrysemys*. Moreover, *dorsalis* is known to have a much shorter period of tolerance for anoxia than the other morphotypes (Ultsch et al. 1999; Ultsch et al. 1985), hinting at adaptation to long winters in *picta*, *bellii* and *marginata*, the three more northern morphotypes.

It is often lamented that the application of various species concepts results in the recognition of vastly different numbers and identities of taxa (Agapow et al. 2004; Isaac et al. 2004). The genus *Chrysemys* appears to be no different and the difficulties may indeed be exacerbated

by the above mentioned complications. Adherence to the biological species concept (BSC), which defines species boundaries based on the criteria of reproductive isolation (Mayr 1969) would support the continued description of *Chrysemys* as a monotypic genus with geographic variation. The existing subspecies (*C. p. picta*, *C. p. dorsalis*, *C. p. bellii*, and *C. p. marginata*) were described based on this geographic variation in morphology and do not have reproductive isolation. The taxonomic unit of subspecies can be controversial as there are no rigorous criteria for designating them, and so many taxonomists do not support the recognition of those units (Zink 2004).

The phylogenetic species concept (PSC, Cracraft 1983) endeavours to define the barrier between the reticulate relationships that occur within a population and the hierarchical relationships among divergent lineages; individuals that are related through genealogical patterns of ancestry and descent, as opposed to nested and divergent lineages are part of the same phylogenetic species. Operationally, delimitation is done based on the criteria of fixed differences between phylogenetic species that can be used for diagnosis, often identified through population aggregation analysis (Davis and Nixon 1992). Adherence to the PSC would support the description of *C. dorsalis* and *C. picta* (encompassing *picta*, *bellii* and *marginata*) based on the five fixed mtDNA CR characters that can be used to diagnose the membership of an individual to one of the phylogenetic species. A common critique of the PSC is that fixed differences can quickly evolve, especially when population sizes are small, resulting in too many entities being described (Agapow et al. 2004; Frankham et al. 2012). Additionally, having sufficient sampling that is representative of the whole range of a species is important to determine which characters are truly fixed and different. In this study I

intentionally included only individuals that were clearly assigned to a morphotype, avoiding geographically intermediate sampling locations. The inclusion of individuals from other areas, particularly in the regions where the ranges of the morphotypes overlap, could falsify the diagnostic ability of the characters reported here.

The genealogical species concept (GSC) defines species on the basis of forming monophyletic entities in cladistics analysis (Baum and Shaw 1995). The GSC is used by Starkey et al. (2003) when interpreting the two monophyletic entities reconstructed in their tree-based analyses, thus resulting in the elevation of *dorsalis* to the species level as sister to *C. picta* (encompassing *picta*, *bellii* and *marginata* subspecies). However, in the tree based analyses in this study (Figure 3.4), *dorsalis* does not consistently form a monophyletic group, sister to the rest of *Chrysemys*, making the outcome of adherence to the GSC unclear (see below).

Although the morphotypes *picta*, *marginata* and *bellii* are not diagnosable by either fixed genetic characters or monophyletic entities, there are trait frequency differences that differentiate them. This finding is contrary to the suggestion by Starkey et al. (2003) that a continuously shared gene pool exists within what they call *C. picta*. There are large and significant F_{ST} values between each of the morphotypes, and the AMOVA shows that there is more genetic variation among than within the morphotypes, even when the potentially more diverged *dorsalis* is excluded from the analysis. This result suggests that each morphotype may represent a local genealogical unit, in which most genetic recombination occurs. Neither the BSC or GSC lends such units formal distinction, however, Davis and Nixon (1992) do

support the acknowledgement of such local genealogical units under the PSC (but see Cracraft 1983; Eldredge and Cracraft 1980; Nelson and Platnick 1981; Nixon and Wheeler 1990).

3.4.2 Discordance among results

The addition of a nuclear gene does not appear to be of much assistance in resolving *Chrysemys* taxonomy. In contrast to the CR, there are no fixed differences in the PAX-P1 intron that can be used to diagnose any of the four morphotypes and Bayesian inference methods returned a poorly resolved gene tree. Whether this lack of informativeness is due to an absence of phylogenetic signal in PAX-P1 or discordant patterns between mtDNA and nuDNA is unclear. Other studies using nuclear markers to resolve relationships within the Emydidae found many nuclear genes show little informative variation in this group (Wiens et al. 2010). Alternatively, there is a large body of literature showing that mtDNA and nuDNA frequently return discordant patterns with various potential explanations (Fisher-Reid and Wiens 2011; Toews and Brelsford 2012). For this reason, many authors recommend using several nuclear genes as well as mtDNA in phylogenetic studies (Zink and Barrowclough 2008). My study was limited to the use of a single nuclear region because preliminary evaluations of other nuclear regions (*c-mos,* R-35 and GAPD, data not shown) did not find them to be promising candidates for being phylogenetically informative. I initially targeted the PAX-P1 intron as it is highly informative at or around the species boundary in other chelonian taxa (Spinks et al. 2012a; Spinks et al. 2012b, Garrick et al. submitted). In contrast to the relatively deeper signal typical of other nuclear introns, PAX-P1 has been shown to

exhibit levels of variation similar to the mtDNA CR, suggesting that it may complement the CR in reconstructing relatively recent population history.

There is also discordance in the relative relationships reconstructed by the various tree-based analyses (Figure 3.4). This result could be due to weak phylogenetic signal in the gene sequences or the inappropriate application of cladistic methods. As is cautioned by Goldstein et al. (2000), applying cladistic analysis when the underlying relationships among terminals are not-hierarchical may force a spurious hierarchical result. The various topologies produced by the different optimality criteria and highly mobile placement of *dorsalis* may be an indication that a hierarchical structure was improperly imposed upon the data set. Although there is little congruence across the tree-based analyses in this study regarding the placement of *dorsalis*, the results of the neighbour joining tree in this study (Figure 3.4 B) are consistent with that of Starkey et al. (2003), which position *dorsalis* as monophyletic and basal with respect to the rest of *Chrysemys*. However, the neighbour joining analysis method will always produce a fully resolved tree and may represent the worst case of inappropriately applying hierarchical methods. The Bayesian method allows for polytomies (unresolved branches) and only shows branching where there is sufficient support for that event. Thus, although the neighbour joining tree indicates the relative relationships among the terminals in more detail, those relationships may not have much support and are better displayed as polytomies, as in the Bayesian analysis, which does not force as many hierarchical nodes.

If relationships among the terminals in the analysis are non-hierarchical, then the network analyses should display the reticulating relationships more accurately, including allowing for

the presence of extant ancestral nodes, and the proper depictions of multifurcations and reticulation due to homoplasy (Posada and Crandall 2001). It is possible that all three of those circumstances occur within the CR data set, with the additional situation of potential recombination in PAX-P1, suggesting that the network-based analyses are more appropriate for reconstructing the evolutionary history of *Chrysemys*.

The CR network presented here (Figure 3.2) shows *dorsalis* to have six unique haplotypes that are separated from all other sampled haplotypes by at least eight character changes. Morphotype combinations *picta-marginata*, and *marginata-bellii* share haplotypes, with any haplotypes unique to a morphotype often interspersed in the network, particularly for *marginata* and *picta*. With additional sampling of individuals, it is possible that many of the haplotypes that are unique to a morphotype in this study would be found to be shared among morphotypes. The high degree of haplotype sharing and general lack of segregating, morphotype-specific haplotypes indicates that historical relationships among *picta*, *bellii* and *marginata* are more reticulating than hierarchical. The PAX-P1 network (Figure 3.3) is also highly reticulating, with no clear segregation by morphotype. In fact, reconstructed PAX-P1 haplotypes are shared among all combinations of morphotypes except *dorsalis – marginata*, further indicating reticulating rather than hierarchical relationships.

3.4.3 Implications for *Chrysemys* paleogeographic history and conservation

Taxonomy aside, there are several other interesting results from this study. Starkey et al. (2003) commented on how the recent postglacial expansion of *bellii* is reflected in the low genetic diversity detected within that subspecies. Here, I find *bellii* to have lower haplotypic

diversity than any of the other subspecies, particularly at the PAX-P1 intron, which supports some of the assertions of Starkey et al. (2003). They postulated that a possible period of extreme aridification in the Great Plains/Rocky Mountain region that was maximal 14000 years ago [proposed by Bartlein et al. (1998)] might have limited the range of *Chrysemys* to eastern North America. Due to the Wisconsinan glaciation, *bellii* could have arrived in the most north-western part of their range in BC after 12000 years ago, and potentially much later if expansion west of Chicago was not possible prior to 14000 years ago (Starkey et al. 2003). The relatively lower levels of genetic diversity detected in that part of the range are consistent with these paleogeographic reconstructions. Yet, in BC, there are unique CR (B.1 and B.2) and PAX-P1 haplotypes (B.4 and B.5), indicating that those peripheral populations may contain evolutionary novelty important for local adaptation.

Although *Chrysemys picta* and its currently recognized subspecies are listed globally as "least concern" by the IUCN (Van Dijk 2011), in BC, the western painted turtle is considered to be Endangered or of Special Concern in different areas (COSEWIC 2006). Controversy can arise when species are listed as of conservation concern when they are locally rare within a political jurisdiction, yet are globally common (Hunter and Hutchinson 1994). Evidence that BC populations have genetic diversity that is unique within the species suggests that conservation efforts in BC may be warranted (see Chapter 2).

3.4.4 Summary

It is clear that resolving the taxonomy of *Chrysemys* will depend upon which species concept is applied. No genetic evidence was found in the nuclear gene to support the elevation of *dorsalis* to the species level; however the number of fixed differences in the mitochondrial gene does indicate lineage separation. The mobile phylogenetic placement of *dorsalis* relative to the rest of *Chrysemys* is a source of uncertainty regarding the strength of evidence for *dorsalis* as a basal rather than derived lineage from *Chrysemys*. However, the additional data and analyses conducted here do not provide compelling evidence to refute the tentative designation of *C. dorsalis* and *C. picta* (encompassing *picta*, *bellii* and *marginata*) as separate species, as has been provisionally adopted by the Society for the Study of Amphibians and Reptiles (Crother 2012) and the Turtle Taxonomy Working Group (Rhodin et al. 2010). The existence of hybrid zones is problematic to the interpretation of the results of this study, as individuals from those regions were excluded from these analyses given lack of accompanying morphological identification to subspecies. Further studies including increased sampling of individuals from the entire range of *Chrysemys* and a larger sample size of *dorsalis* in particular, using a suite of nuclear markers will be necessary to shed further light on the taxonomy of this group. The recent publication of the genome of *C. p. bellii* (Abramyan et al. 2013) could help identify single nucleotide polymorphisms (SNP) and genes that may have neutral and adaptive differences to target in future studies.

4 Chapter: CONCLUSIONS

4.1 Research findings and significance

This is the first genetic assessment of *Chrysemys picta bellii* in British Columbia, and to my knowledge, the first detailed population genetic study for this group overall, representing a significant contribution towards informing the best management for this species-at-risk in BC. This research shows that BC western painted turtles contain both nuclear and mitochondrial genetic diversity that is unique within the species, despite BC constituting just a small portion of the species' range. The insights gained by applying two different conservation unit criteria to western painted turtles in BC constitute a more thorough assessment of the genetic diversity within BC turtles than either concept alone. From the ESU approach, it is clear that BC turtles are closely linked evolutionarily to the western painted turtles in other parts of North America, which is consistent with their recent expansion into western North America and BC. The ESU method also indicates that processes at the site level are important to monitor to ensure population persistence, as each occupied site included in this study is differentiated and demographically independent from all others. The DU approach highlights the regional nature of genetic diversity patterns and forces reflection as to the ecological significance of differentiation at the regional level. Although the ESU and DU conservation unit concepts result in the recommendation of different units, they are complementary in the case of the western painted turtle in BC, indicating the existence of genetic diversity at the site, regional and range wide levels.

Anecdotal reports suggesting that western painted turtles may have been introduced to the coast from sites in the interior in the recent past (COSEWIC 2006) have been clouding conservation efforts in that region. I do not find genetic evidence to support such reports, and instead find that all of the coastal sites included in this study are part of genetic clusters that are unique to that area. The genetic clusters identified are geographically cohesive, despite it being difficult to infer the processes that would lead to the Sunshine Coast-Gulf Island-Vancouver Island (SGV) grouping. The geographic cohesiveness of the clusters indicates that the presence of turtles in each region is due to natural processes, not recent human introductions. It is more difficult to determine whether the occupation of a particular site is natural or due to human movement of turtles within a region. There are some sites in the lower mainland that were excluded from the analyses in Chapter 2 due to very small population sizes (n=1-2). Preliminary results from assignment tests indicate that some of these turtles may have been translocated from the interior (data not shown), providing some evidence that sites occupied by only two or three turtles may be due to human activities. It is only because I now have a reference data set for genetic diversity across the regions of BC that such assignment tests are possible, which will become a valuable asset in the future to determine the origin of turtles if controversy over their source arises. In summary, there is no evidence to support the anecdotal reports that western painted turtles are not native to the coast; furthermore, although there are likely some individuals at sites in the lower mainland that originated from the interior, this does not appear to be a widespread occurrence.

Human-mediated introduction of non-native turtles is an additional concern for western painted turtle populations in urban settings. For example, there is ongoing monitoring of red

eared slider (*Trachemys scripta elegans*) populations, as they may pose a threat to the native western painted turtles through increased competition (Bunnell 2005). Here, I find that the introduction of painted turtles from other areas of North America to Burnaby Lake is also a serious threat to the native genetic diversity at that site since hybridization between native and non-native painted turtles has now been documented. It is unclear where the non-native painted turtles originated and how they came to be introduced at Burnaby Lake. For this reason monitoring for non-native painted turtles should be ongoing at other lakes in the lower mainland of conservation significance.

Despite carrying out the most thorough genetic investigation regarding *Chrysemys* taxonomy to date using both nuclear and mtDNA markers, I am unable to resolve the uncertainty regarding whether *dorsalis* should be considered a separate species from other painted turtles. The tentative conclusion of lineage separation between *dorsalis* and the rest of *Chrysemys* found by Starkey et al. (2003) based on mtDNA stands after the inclusion of samples in BC and Ontario in this study, although based on different lines of evidence. The tree-based analyses were inconclusive as to the placement of *dorsalis*, but the existence of five fixed character differences in the CR between *dorsalis* and the rest of *Chrysemys* serves as evidence of lineage separation. This result, however, should be interpreted with caution due to the possibility of overdiagnosis resulting from the lack of detailed morphological data associated with available range-wide samples. The nuclear intron, PAX-P1, does not show lineage separation among any of the morphotypes. Nevertheless, there is frequency based evidence that suggests that the morphotypes may exist as local genealogical units based on both the CR and PAX-P1. Whether the lineage separation in the CR between *dorsalis* and the

rest of *Chrysemys* is sufficient to warrant describing them as separate species remains unclear, as does whether the shallow, frequency based differentiation of the morphotypes *picta*, *bellii* and *marginata* should be recognized formally.

4.2 Limitation of this study and future work

A genetic assessment is only as good at the number and distribution of samples analyzed. Within BC, I sampled the vast majority of the known range of the western pained turtle, but there are a few key sites that would have contributed greatly to this study, if samples had been available. One such site is Naniamo, on Vancouver Island, which could help determine the boundary between the South Coast and the SGV genetic clusters. Samples from the lower Sunshine Coast could also help determine the boundary between the South Coast and Sunshine Coast genetic clusters on the main land. Moreover, there is very little known about whether occupied sites exist in the area between Kamloops and Williams Lake. Samples from any intervening populations, if present, would help determine the extent of the Cariboo genetic cluster. Although I do have samples from Burning Creek, near 150 Mile House, I was unable to incorporate them in the assessment in Chapter 2 due to poor sample quality (see Appendix B); however, further effort to genotype these individuals could be made.

For the samples from outside of BC, I must rely on second hand information from collaborators regarding the exact site of origin for each sample. Knowing the exact location for each sample is important, as it was the geographic location of sampling that was used in conjunction with a coarse range map to determine the morphotype/subspecies of each individual for use in the analyses in Chapter 3 and Appendix A. Because the ranges of the

morphotypes are not well documented, I was conservative when choosing individuals for the population aggregation analysis in Chapter 3 and only included individuals that were sampled well within the core of the range of the morphotype. Excluding individuals from near the overlapping parts of the morphotypes' ranges does potentially overestimate the number of fixed differences between morphotypes. In order to include more individuals in those analyses, I would need detailed information about the morphology of each individual and whether the water body sampled in contains individuals from more than one morphotype. Including individuals from the entire range of *Chrysemys* and increased numbers of samples from each morphotype will be important for any future studies.

Equally important to the geographic sampling of a study such as this one is the sampling across the genome. The tests for linkage among microsatellite loci indicate that each of the nine loci are inherited separately and represent independent samples of the nuclear genome. Also, the microsatellite loci do not show patterns that deviate from neutral expectations, as would be caused by linkage to loci under natural selection. The inclusion of additional microsatellite loci would have enriched the analyses, however, nine loci is within the range typically used in studies such as this (Coleman et al. 2013; Echelle et al. 2009). The CR is a commonly used mitochondrial marker for turtles, which has been found to provide better phylogenetic resolution than other mitochondrial regions (Lamb et al. 1994). As mtDNA is inherited without recombination, phylogenetic patterns should be consistent across the entire mitochondrial genome and the use of multiple regions is not necessary provided that the region used is sufficiently variable. As discussed in Chapter 3, additional nuclear markers will be needed to further address the taxonomic uncertainty in *Chrysemys*. I initially screened

four nuclear introns for use in this study, however, RNA fingerprint protein 35 intron 1 (Fujita et al. 2004) and Glyceraldehyde-3-phosphate dehydrogenase (GAPD) XI intron (Friesen et al. 1997) were found to be less variable in a pilot sample than the *c-mos* oncogene and PAX-P1 introns. The *c-mos* oncogene was subsequently sequenced for a larger number of individuals for use in the Burnaby Lake study (Appendix A), but was found to have weak phylogenetic signal (data not shown). Although the phylogenetic signal also appears to be weak in PAX-P1, that marker has been successfully used in other chelonian taxa (Spinks et al. 2012a; Spinks et al. 2012b, Garrick et al. submitted), and was a reasonable candidate for use in this study. In depth screening of additional informative nuclear regions is warranted and required for future studies to be successful in resolving the taxonomy and relationships within *Chrysemys*.

The recent publication of the genome of a *C. p. bellii* individual (Abramyan et al. 2013) could allow the development of molecular tools to investigate adaptive differences among painted turtles. Genetic markers that are linked to adaptive differences can be used both to clarify taxonomy and delineate and prioritize conservation units (Bonin et al. 2007; Funk et al. 2012; Ouborg et al. 2009; Reed and Frankham 2001). Information regarding the potential adaptive differences among the six genetic clusters identified in Chapter 2 could resolve the uncertainty about whether the clusters meet the significance criteria of the DU concept. Similarly, information regarding adaptive differences among the four morphotypes of *Chrysemys* could be used to clarify whether any of the morphotypes are sufficiently differentiated to merit recognition as a separate evolutionary entity.

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Appendices

Appendix A : Investigating the genetic consequences of introduced *Chrysemys picta* on endangered *C. p. bellii* in British Columbia

A.1 Background

The introduction and establishment of non-native individuals can impose severe impacts to native fauna, especially if interbreeding with the resident form occurs, resulting in hybridization and genetic introgression (Rymer and Simberloff 1996; Huxel 1999). A common source of non-native individuals is often the release of unwanted pets as in the case of turtles (Stuart and Parham 2007). Populations with genetic contamination through introgression may be compromised from a conservation standpoint, so the use of genetic methods to detect non-native and hybrid individuals can be important to inform management (Allendorf et al. 2001).

The western painted turtle (*Chrysemys picta bellii*) is listed as Endangered in the Pacific Coastal designatable unit in British Columbia (COSEWIC 2006). It is the only extant native pond turtle found in Western Canada and for that reason, holds special ecological and cultural significance (COSEWIC 2006). In the lower mainland of BC, Burnaby Lake (Figure A.1) is an important stronghold of the species and is one of only two sites in that region where recruitment has been documented (A. Mitchell, personal communication). In 2010, volunteers watching for nesting on a newly created artificial nesting beach observed six females nesting that displayed colour patterns on their plastrons that are atypical of the native

C. p. bellii (Figure A.2a and A.2b), and more similar to non-native conspecific subspecies found in other parts of the wide range of this species (A. Mitchell, personal communication).

Figure A.1 Map of Burnaby Lake.

Oval encompasses the area at the east end of the lake where 23 turtles were sampled, including at the artificial nesting beach. Square encompasses the area at the west end of the lake where two turtles were sampled

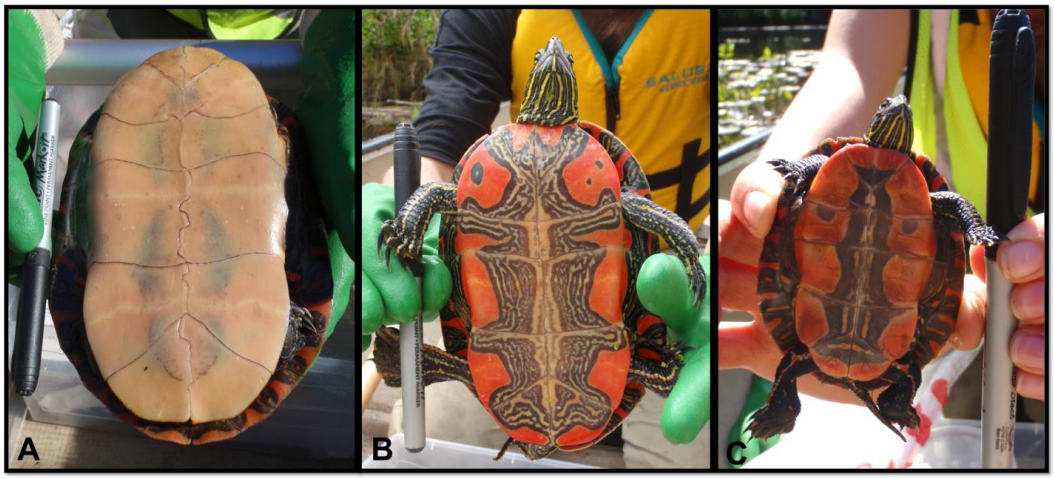

Figure A.2 Morphology of native and non-native painted turtles.

a. Turtle with non-native morphology sampled at Burnaby Lake, *b.* Example of a typical western painted turtle, *c.* Juvenile sampled at Burnaby Lake with western painted turtle morphology and a non-native control region haplotype

The distribution of *C. picta* is continuous across the North American continent, with geographic variation recognized in the form of four subspecies (*C .p. picta, C. p. marginata, C. p. dorsalis,* and *C. p. bellii*) with overlapping ranges (Ernst and Lovich 2009). Hybridization is common where the ranges overlap (Weller et al. 2010), which is cause for concern if non-native conspecifics are now present in Burnaby Lake. The introduction of alleles from elsewhere could be detrimental to the local populations if those alleles are maladaptive to the local conditions, resulting in a decrease in fitness due to outbreeding depression (Allendorf et al. 2001). Also, genetic contamination could affect the conservation value of introgressed populations (Allendorf et al. 2001).

Previous phylogeographic analyses did not find sufficient differentiation at the mitochondrial DNA (mtDNA) control region (CR) to warrant continued recognition of *picta*, *marginata* and *bellii* as separate evolutionary lineages (Starkey et al. (2003), however these range-wide haplotypes are informative for determining coarse geographic origin (see Chapter 3). However, mtDNA is uni-parentally inherited and thus limited to reconstructing matrilines. To complement insights from mtDNA, nuclear genotypic data have proved useful for investigating the presence and direction of hybridization, and degree of introgression in a wide range of species (Mikulicek et al. 2012; Gonzalez-Trujillo et al. 2012; Biedrzycka et al. 2012; Vilaca et al. 2012). Here, I used mitochondrial and nuclear DNA sequence data to investigate whether individuals with atypical *C. p. bellii* morphology in Burnaby Lake are non-native, and to reconstruct their likely origin. Further, I used nuclear microsatellite genotypic data to estimate relatedness among introduced individuals in order to infer whether their presence is due to release from a single or multiple sources.

A.2 Methods

Site and sampling

Burnaby Lake is a large post-glacial lake in an urban setting within the city of Burnaby in the lower mainland of BC (Figure A.1 and A.3). It is a popular lake for recreational activities and is easily accessible on all sides via a walking trail that circles its perimeter. Blood samples (~100µl) were collected from the dorsal coccygeal vein from 24 adults (plastron length >10 cm) and one juvenile (plastron length <7 cm) painted turtle at Burnaby Lake. Twelve samples were collected from females following nesting at the artificially created nesting beach at the east end of Burnaby Lake; the other 13 turtles were collected by dip netting (Figure A.1). Only two samples were collected from the west end of Burnaby Lake (Figure A.1). Blood was stored in tubes containing a buffer solution (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS; Longmire et al. 1997) and refrigerated until analysis. Of the adult turtles, 12 individuals qualitatively displayed typical *C. p. bellii* morphology whereas the other 12 displayed morphology which, to varying degrees, more closely resembled other subspecies of *C. picta* (Figure A.2, E. Jensen, personal observation). Samples were collected from turtles (n=26) from 12 other lakes in BC as part of a larger study assessing genetic diversity of *C. p. bellii* in BC (see Chapter 2) and were used in this study as references for native BC genotypes (Figure A.3, Table A.1). Tissue samples from across North America (n=46) were obtained from the archives of Dr. Bradley Shaffer (University of California, Los Angeles) and the Queen's University Biological Station to be used as references for the other subspecies (Figure A.3, Table A.1).

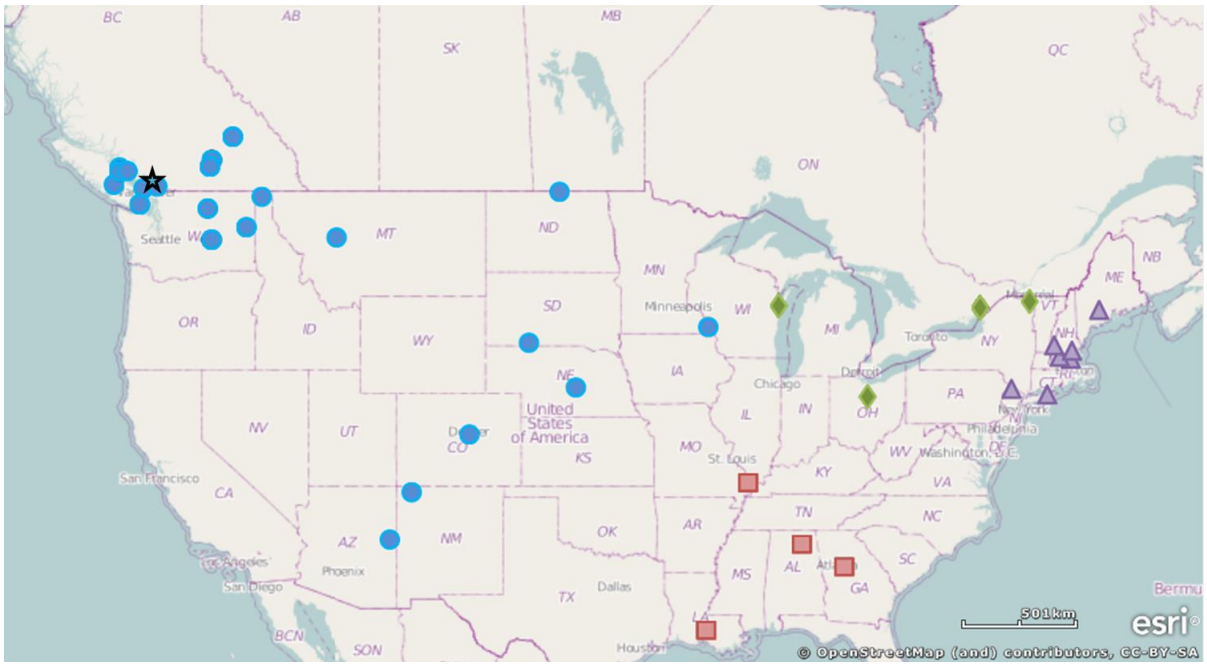

Figure A.3 Map of the localities of *Chrysemys* individuals for which the *c-mos* oncogene was sequenced.

The star indicates the location of Burnaby Lake. Blue circles are *C. p. bellii*, red squares are *C. p. dorsalis*, green diamonds are *C. p. marginata* and purple triangles are *C. p. picta*.

Table A.1 Source and locality information for *Chrysemys* samples for which *c-mos* was sequenced

PS present study

Voucher ID	Source	State/ Province	County /Region
HBS27284	Shaffer	AL	Marshall
HBS27288	Shaffer	AL	Marshall
Alak.5.12	PS	BC	Lower Mainland
Alak.51	PS	BC	Lower Mainland
Burn.21	PS	BC	Lower Mainland
Burn.212	PS	BC	Lower Mainland
Burn.213	PS	BC	Lower Mainland
Burn.214	PS	BC	Lower Mainland
Burn.2140	PS	BC	Lower Mainland
Burn.215	PS	BC	Lower Mainland
Burn.217	PS	BC	Lower Mainland
Burn.218	PS	BC	Lower Mainland
Burn.219	PS	BC	Lower Mainland
Burn.22	PS	BC	Lower Mainland
Burn.223	PS	BC	Lower Mainland
Burn.224	PS	BC	Lower Mainland
Burn.225	PS	BC	Lower Mainland
Burn.226	PS	BC	Lower Mainland
Burn.227	PS	BC	Lower Mainland
Burn.23	PS	BC	Lower Mainland
Burn.24	PS	BC	Lower Mainland
Burn.25	PS	BC	Lower Mainland
Burn.26	PS	BC	Lower Mainland
Burn.27	PS	BC	Lower Mainland
Burn.28	PS	BC	Lower Mainland
Burn.29	PS	BC	Lower Mainland
Burn.32	PS	BC	Lower Mainland
Burn.35	PS	BC	Lower Mainland
Burn.56	PS	BC	Lower Mainland
GBHNR.1	PS	BC	Lower Mainland
SL.33	PS	BC	Lower Mainland
SL.37	PS	BC	Lower Mainland
OF.26	PS	BC	Okanagan
OF.27	PS	BC	Okanagan
OF.29	PS	BC	Okanagan
OF.30	PS	BC	Okanagan
RE.1	PS	BC	Okanagan
RE.2	PS	BC	Okanagan
RE.3	PS	BC	Okanagan
RE.4	PS	BC	Okanagan
Cran.51	PS	BC	Sunshine Coast
WE.318	PS	BC	Sunshine Coast
WE.34	PS	BC	Sunshine Coast
TI.113	PS	BC	Texada Island
TI.23	PS	BC	Texada Island
RV.32	PS	BC	Thompson
RV.85	PS	BC	Thompson
RV.98	PS	BC	Thompson
BE.136	PS	BC	Vancouver Island
BE.137	PS	BC	Vancouver Island
BE.17	PS	BC	Vancouver Island
PA.24	PS	BC	Vancouver Island
PA.28	PS	BC	Vancouver Island
HBS27533	Shaffer	CO	Douglas
HBS28620	Shaffer	CO	LaPlata
HBS26213	Shaffer	GA	Jackson
HBS28303	Shaffer	ID	Boundary
HBS23169	Shaffer	IL	Alexander
HBS23170	Shaffer	IL	Alexander
HBS28134	Shaffer	KS	Sherman
HBS31533	Shaffer	LA	St Martin
HBS26058	Shaffer	MA	Middlesex
HBS33050	Shaffer	MA	Worcester
HBS28035	Shaffer	ME	Kennebec
HBS28041	Shaffer	ME	Kennebec
HBS23616	Shaffer	MN	Houston
HBS28277	Shaffer	MT	Lewis and Clark
HBS27748	Shaffer	ND	Rolette
HBS28049	Shaffer	NH	Salem
HBS28026	Shaffer	NH	Sullivan
HBS31658	Shaffer	NJ	Sussex
HBS28644	Shaffer	NM	Apache
HBS28625	Shaffer	NM	San Juan
HBS28001	Shaffer	NY	Clinton
HBS27581	Shaffer	NY	Sufflolk
HBS27179	Shaffer	OH	Shelby

Data collection and analysis

DNA was extracted from blood samples using the Nucleospin QuickBlood kit (Macherey-Nagel) following manufacturer's protocols. DNA was extracted from tissue samples using the Nucleospin Tissue kit (Macherey-Nagel) following manufacturer's protocols.

Mitochondrial sequences

A 671 base pair segment of the mitochondrial genome (mtDNA), including part of the control region (CR) was amplified as a single fragment using the methods described in Chapter 2. Exemplar CR sequences from across North America were taken from the Starkey et al. (2003) popset in GenBank (accession number JQ963656). All the CR sequences were aligned in Geneious (Biomatters Ltd.) using Geneious Aligner and a haplotype network was generated using statistical parsimony, as implemented in TCS (Clement et al. 2000).

Nuclear sequences

A 519 base pair segment of the nuclear oncogene *c-mos* (Saint et al. 1998) was sequenced for all Burnaby Lake individuals (n=25) and a subset of BC individuals (n=26) as well as for a representative sample (n=45) of individuals from across North America (Table A.1). The CMOSG77 primer from Saint et al. (1998) was used in conjunction with Cp_CMOSG78 (5′-AGGGTGATGTCAAAGGAGTAGATGTC-3´; this study) in 25 µl reactions containing ~20-40 ng DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 7.5µg bovine serum albumin, 0.4µM each primer and 0.5 U KAPA Taq DNA Polymerase. Cycling

conditions consisted of 94˚C for 2 minutes, 35 cycles of 95 ˚C for 30 seconds, 55 ˚C for 30 seconds, 72 ˚C for 45 seconds, and a final extension at 72 ˚C for 7 minutes. The sequencing reactions were performed using ABI BigDye v3.1 Terminator chemistry and sequences were run on an Applied Biosystems 3130XL DNA automated sequencer. Sequences were visualized and edited using Sequencher 5.0 (Gene Codes Corporation). The *c-mos* sequences were aligned using Geneious Aligner and examined for polymorphisms.

Microsatellites

The Burnaby Lake individuals were genotyped at ten microsatellite loci (Table B.2) (Gonçalves da Silva et al. 2009; Hauswaldt and Glenn 2003; King and Julian 2004; Pearse et al. 2001) using the methods described in Chapter 2. A high level of missing data were found at locus GT124 (48%), which was subsequently excluded from further analysis. Locus Cp2 was also excluded for the reasons discussed in Chapter 2. The pairwise relatedness (Queller and Goodnight 1989) among Burnaby Lake individuals was estimated in GenAlEx (Peakall and Smouse 2006).

A.3 Results

Mitochondrial haplotypes

Four mtDNA CR haplotypes were detected across the representative sampling from BC, each different by only a single base pair change (Figure A.4). All individuals identified as non-native based on morphology were found to have CR haplotypes common to eastern North

America and differing from BC native haplotypes by at least four character changes. Of those 12 individuals, 11 shared a single haplotype (haplotype S.3, Figure A.4). The other morphologically non-native individual had a different haplotype (haplotype S.4, Figure A.4). Haplotype S.4 is found in *C. p. bellii* individuals, however only those sampled at the eastern-most portion of the range in Wisconsin, Illinois and Minnesota. The only juvenile turtle to be sampled at Burnaby Lake, which was morphologically identified as native (Figure A.2c), had the more common of the non-native CR haplotypes, S.3.

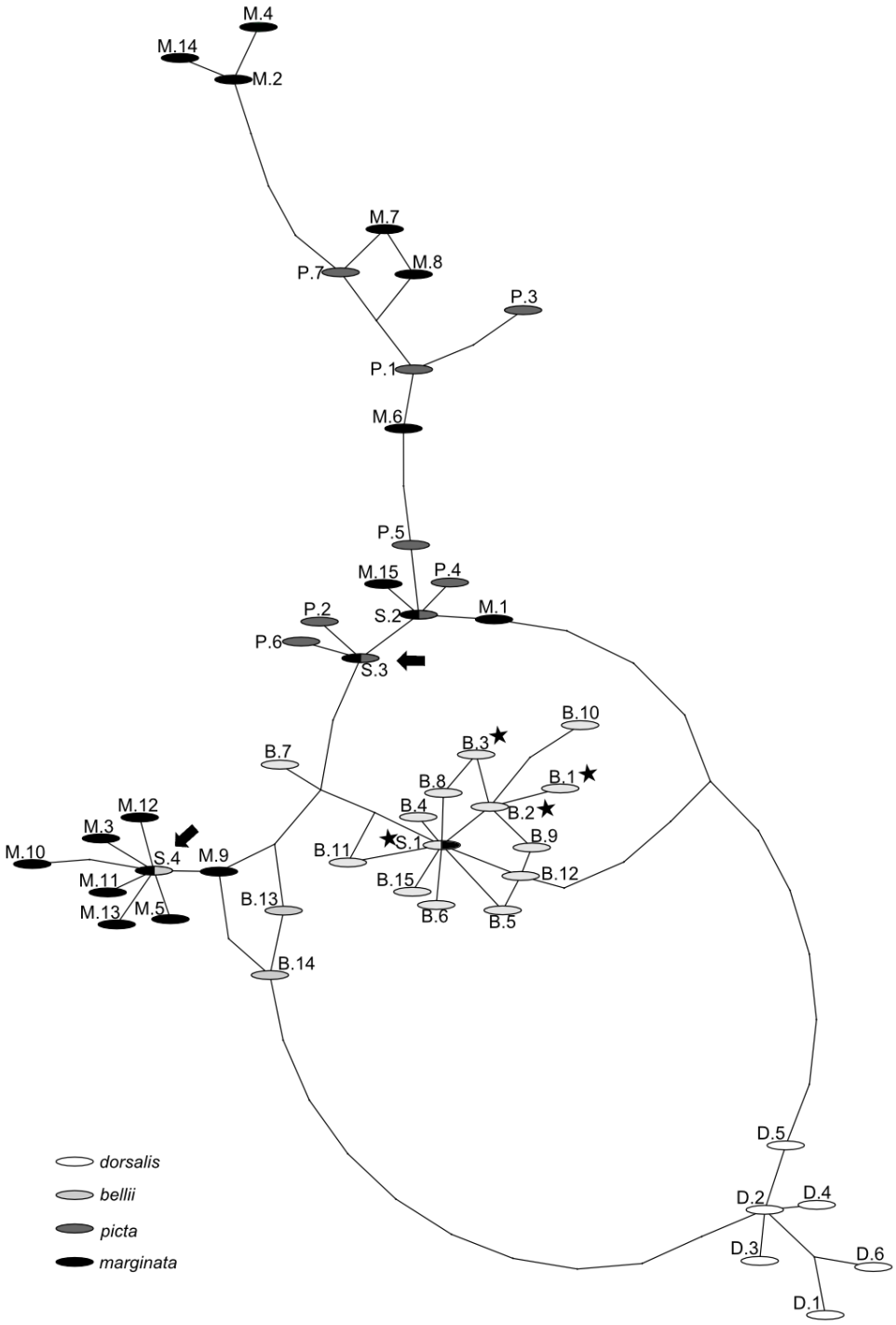

Figure A.4 Haplotype network reconstructed based on the mtDNA control region data

Ovals indicate haplotypes in the sample. Single colour ovals are those found in only one subspecies, dual coloured nodes are those detected in more than one subspecies. Frequencies of the shared haplotypes are not indicated. Inferred but unsampled nodes are indicated as links in the network. Stars indicate haplotypes that were sampled in native BC individuals; arrows indicate haplotypes that were sampled in the morphologically non-native turtles in Burnaby Lake

Nuclear genotypes

Most of the non-native individuals (75%) had private alleles at two positions in the *c-mos* sequence, *c-mos 205 and c-mos 436* (Table A.2) relative to other turtles in BC. Where all individuals from other sites in BC and all *C. p. bellii* from the rest of their range were monomorphic at these two positions, nine of the non-native individuals were polymorphic for both the native allele and a foreign one. Using the samples from across North America as a reference, the non-native alleles for those positions were found only in individuals belonging to *C. p. dorsalis* and *C. p. marginata* from the USA (Table A.2).

Microsatellites

The relatedness among the morphologically atypical individuals was low (mean -0.118) as estimated from the microsatellite genotypic data. One potential parent-offspring or full-sibling pair (individuals 2-12 and 2-17) was identified, with a pairwise relatedness of 0.513. In addition, the one sampled juvenile turtle with native morphology and a non-native CR haplotype did exhibit a pairwise relatedness value (0.377) and multi-locus allele transmission pattern (data not shown) consistent with a parent-offspring relationship with one of the putatively non-native adults (individual 2-1), suggesting a hybrid origin.

Table A.2 Summary of putative status based on morphology, sex and age class are indicated where known, mtDNA CR haplotype and alleles at two sites in the *c-mos* oncogene

UK unknown

Locality	Individual	Sex	Putative status	CR Haplotype	*c-mos*205	*c-mos*436
BC other (n=26)	-	-	*bellii*	B.1, B.2, B.3, S.1	G	C
Burnaby Lake	2-2	F	*bellii*	B.2	G	C
	2-3	F	*bellii*	B.2	G	C
	2-5	F	*bellii*	B.2	G	C
	2-6	F	*bellii*	B.2	G	C
	3-2	UK	*bellii*	B.2	G	C
	2-140	M	*bellii*	B.1	G	C
	2-19	UK	*bellii*	B.1	G	C
	2-27	M	*bellii*	B.1	G	C
	2-7	F	*bellii*	B.1	G	C
	2-8	F	*bellii*	B.1	G	C
	3-5	UK	*bellii*	B.1	G	C
	5-6	M	*bellii*	B.1	G	C
	2-23	UK	Atypical	S.4	R	Y
	2-1	F	Atypical	S.3	R	Y
	2-12	F	Atypical	S.3	R	Y
	2-13	F	Atypical	S.3	G	C
	2-14	F	Atypical	S.3	R	Y
	2-15	F	Atypical	S.3	G	C
	2-17	UK	Atypical	S.3	R	Y
	2-18	M	Atypical	S.3	R	Y
	2-24	UK	Atypical	S.3	R	Y
	2-25	Juvenile	*bellii*	S.3	R	Y
	2-26	UK	Atypical	S.3	R	Y
	2-4	F	Atypical	S.3	G	C
	2-9	F	Atypical	S.3	G	C
USA			*dorsalis* (n=5)		R	Y
			marginata (n=3)		R	Y
			bellii (n=20)		G	C
			picta (n=9)		G	C
Ontario			*marginata* (n=8)		G	C

A.4 Discussion

All morphologically atypical individuals are likely non-native in origin based on the genetic evidence, exhibiting a CR haplotype as well as *c-mos* alleles that are more representative of painted turtles from eastern North America. I was unable to infer the subspecies or locality of origin based on the genetic data, except that the recovered haplotypes and genotypes were not otherwise detected across the western range of *C. p. bellii*. The majority of non-native individuals are genetically unrelated, potentially indicating multiple introduction events from varied sources. Their presence in Burnaby Lake is possibly due to the release of unwanted pets as reported for other species of turtle elsewhere in the lower mainland (Bunnell 2005), although the mode of introduction at Burnaby Lake is currently unknown.

The non-native individuals have been observed nesting at Burnaby Lake, but it was previously unknown whether the offspring were progeny of two non-natives or whether they were hybrids. Here, I show genetic evidence of hybridization between the introduced non-natives and the local population of *C. p. bellii*. The only juvenile turtle that was sampled is a hybrid exhibiting high relatedness and an allele transmission pattern consistent with parent-offspring relationship with individual 2-1, indicating that some level of genetic introgression is present in this population. There is no reason to believe that hybrid offspring will be sterile or otherwise non-viable, as *C. p. belli* and the other subspecies form hybrid swarms where their ranges overlap (Weller et al. 2010). Yet, hybrid offspring may be less fit if they possess genes that are not adapted to their local environment indicative of outbreeding depression (Allendorf et al. 2001). *Chrysemys* subspecies do naturally hybridize where their ranges overlap, however, individuals involved in the crosses originate from the same geographical

area and ecological conditions. Since the non-native individuals at Burnaby Lake likely originated from eastern North America, they may possess gene variants maladapted in BC. Frankham et al. (2011) provide a decision tree for predicting whether outbreeding depression is likely to occur when populations interbreed. In the case of Burnaby Lake, this decision tree would recommend that the non-native and native individuals be kept from interbreeding since outbreeding depression is predicted to occur, as the populations from which the native and non-native turtles originated have been isolated without gene flow for at least 500 years.

The instance of hybridization detected in this study resulted in an individual that had native morphology, which indicates that genetic testing may be required to identify hybrid individuals. Fully half of the individuals sampled in this study were found to be non-native, indicating that, at the east end of Burnaby Lake at least, non-native individuals make up a high proportion of all painted turtles. No non-native turtles were observed or sampled at the west end of the lake, although turtle density appears to be very low in that area (E. Jensen, personal observation). This study is limited by the number of samples that were available, and the demographic groups represented in the current sample. Further study, ideally including a large number of juvenile and hatchling individuals, is necessary to determine the extent of introgression in the Burnaby Lake population of western painted turtles. Here, I present evidence that hybridization has occurred, but without more samples from additional generations of turtles, it is difficult to estimate the proportion of hybrid individuals and the level of introgression in the population. The development of additional nuclear genetic markers that have the power to diagnose non-native individuals would be needed to positively identify hybrid individuals with native mothers and non-native fathers. The

development of such markers is challenging because, although *C. p. bellii*, *C. p. marginata*, *C. p. picta* and *C. p. dorsalis* are recognized as subspecies, no fixed differences at nuclear markers have been identified so far and there is debate as to whether each subspecies actually exists as an evolutionary lineage separate from the others (see Chapter 3).

If the view is taken that the presence of non-native alleles is undesirable in the Burnaby Lake population of *C. p. bellii*, then intensive management over a long period of time will be required to preserve the native gene pool. Initially, the removal of non-native and confirmed hybrid individuals would be required, with genetic testing of individuals for non-native alleles on-going for years or potentially decades afterwards given the potential for sperm storage in this species (Pearse et al. 2001). In order to confirm whether individuals are hybrids, genetic testing will be required as the hybrid detected in this study was morphologically identified as native. Even if an individual displays an atypical morphology, it may simply be displaying natural and native variation rather than a hybrid phenotype, as there is considerable morphological variation among native western painted turtles, particularly in plastron patterning, dorsal stripes and carapace colouration and patterning (E. Jensen, personal observation).

Additional introduction events of non-native individuals could occur in the future as the source of the non-native individuals at Burnaby Lake has not yet been discovered or ameliorated. Monitoring of lakes across the BC lower mainland, including Burnaby Lake, should be on-going to identify sources of introduction events and their potentially impacts on the viability and genetic integrity of this listed species within the region.

Appendix B : Material supplemental to Chapter 2

B.1 Tables

Table B.1 Details about the ecological drainage unit, faunal province (IM- Intermountain, RM- Rocky Mountain, PC- Pacific Coastal), designatable unit (IM-RM Intermountain-Rocky Mountain, PC Pacific Coastal) and coordinates for each site and the mitochondrial control region haplotypes identified at each

Abbreviation	Sampling Site	Ecological Drainage Unit	Faunal Province	Designatable Unit	Latitude	Longitude	mtDNA Haplotypes
BA	Baird Lake	#10 Thompson	IM	IM-RM	50.5692	-118.7928	2
BE	Beaver Lake	#31 Vancouver Island	PC	PC	48.5138	-123.3934	1
BL	Burnell Lake	#5 Okanagan	IM	IM-RM	49.208	-119.612	1
CV	Creston Valley Wildlife Management Area	# 4 Lower Kootenay	RM	IM-RM	49.123	-116.6299	1
DO	Dorothy Lake	#7 Upper Columbia	RM	IM-RM	50.4984	-116.0257	1
EL	Elizabeth Lake	#8 Upper Kootenay	RM	IM-RM	49.4978	-115.793	1, 3
ER	Erie Lake	#1 Columbia - Arrow Lakes	RM	IM-RM	49.1895	-117.3479	1
JO	Johnson Lake	#8 Upper Kootenay	RM	IM-RM	49.9562	-115.7647	3
KL	Klein Lake	#29 South Coastal	PC	PC	49.7294	-123.9699	1
LO	Loon Lake	#8 Upper Kootenay	RM	IM-RM	49.1132	-115.1062	1
NI	Nicomen Slough	#24 Lower Fraser	PC	PC	49.183	-122.114	1
OF	Fipke Lake near Oyama	#5 Okanagan	IM	IM-RM	50.1323	-119.3558	1
PA	Port Alberni	#31 Vancouver Island	PC	PC	49.2426	-124.827	1
RE	Redlich Pond	#5 Okanagan	IM	IM-RM	49.8945	-119.4616	1
RV	Revelstoke Marsh	#1 Columbia - Arrow Lakes	IM	IM-RM	50.9612	-118.1805	1, 2
RO	Rosebud Lake	#1 Columbia - Arrow Lakes	RM	IM-RM	49.048	-117.2678	4
RU	Ruby Lake Lagoon	#29 South Coastal	PC	IM-RM	49.724	-123.9917	1
SI	Scout Island Nature Centre	#9 Middle Fraser	IM	IM-RM	52.1186	-122.1164	1
SC	Stephen Coyote Regional Park	#5 Okanagan	IM	IM-RM	49.9579	-119.4394	1

Abbreviation	Sampling Site	Ecological Drainage Unit	Faunal Province	Designatable Unit	Latitude	Longitude	mtDNA Haplotypes
SH	Shannon Lake	#5 Okanagan	IM	IM-RM	49.8563	-119.612	1
SK	Skmana Lake	#10 Thompson	IM	IM-RM	50.8788	-119.7292	1
SW	Swan Lake	#31 Vancouver Island	PC	PC	48.4636	-123.3733	1
TE	Texada Island	#29 South Coastal	PC	PC	49.7462	-124.545	1
TI	Tie Lake	#8 Upper Kootenay	RM	IM-RM	49.4148	-115.3095	1
WE	West Lake	#29 South Coastal	PC	PC	49.7322	-124.0851	1

Table B.2 Characteristics of the 10 microsatellite loci used in this study

Locus	Primer Sequences (5'-3')	Repeat Unit	Allele Size Range (bp)	PCR Method	M13 Label	Source
CpGT108	CCTAGAAAGTAAGAACCAATTTCAG	(CA)4CT(CA)11	230-336	TD59-51	VIC	Goncalves da Silva et al. (2009)
	CCACCAACAGAAGGAAGTTAGTG					
CpGT124	TCGGGGAGCACACTATACC	(GT)31(GC)5	193-245	TD59-51	PET	Goncalves da Silva et al. (2009)
	CTCAGCCCCAAAATGAAC					
Cp2	CTCTAAGGGTTGCACTTCTCAAA	GT	212-246	TD59-51	FAM	Pearse et al. (2001)
	GAGGTGGCATCAAAACATCAT					
Cp3	ATCTTTAAGTCTGTGAACTTCAGGG	GT	154-192	TD55-45	NED	Pearse et al. (2001)
	CTGTCTCATGCAAAGCTGGTAG					
TerpSH2	TGGCCAGCAGGAGTAATG	AGAT	172-244	TD59-51	PET	Hauswaldt and Glenn (2003)
	CTATTAGGGCAGAGACGAG					
TerpSH3	TCCCCCAATGCACAC	CAAA	291-323	TD55-45	FAM	Hauswaldt and Glenn (2003)
	CTGCCCAATCCATTTAGA					
TerpSH7	CACACACACTGTATTTTGATA	AGAT	117-165	TD59-51	VIC	Hauswaldt and Glenn (2003)
	CTATGCCCTTTCTAGTTTG					
GmuD21	GCAGTTAGGCATTACTCAACATC	ATCT	166-226	TD55-45	VIC	King and Julian (2004)
	AGGGTATGAATACAGGGGTGTC					
GmuD28	AGCTGTTTGTCATCATACACTCTC	ATCT	234-286	TD59-51	NED	King and Julian (2004)
	TGGCCCTCATGTTTTATAAGTG					
GmuD62	GGTGGTATAGAAAATCCTAAAATGG	ATCT	171-215	TD59-51	PET	King and Julian (2004)
	GTGCAAACTGTCTGGAAATAGG					

Table B.3 Genbank accession numbers and haplotype for each sequence used in the haplotype analysis

Voucher	Accession #	Haplo-type
HBS27532	JQ963802.1	3
HBS27533	JQ963803.1	3
HBS28092	JQ963838.1	3
HBS28097	JQ963839.1	3
HBS28101	JQ963840.1	3
HBS28134	JQ963844.1	3
HBS28138	JQ963845.1	3
HBS28146	JQ963846.1	3
HBS28171	JQ963847.1	3
HBS28603	JQ963861.1	3
HBS28610	JQ963862.1	14
HBS28620	JQ963863.1	14
HBS28535	JQ963859.1	3
HBS28303	JQ963856.1	1
HBS26294	JQ963749.1	3
HBS26298	JQ963751.1	3
HBS28109	JQ963841.1	7
HBS28111	JQ963842.1	3
HBS28113	JQ963843.1	3
HBS23506	JQ963679.1	3
HBS23507	JQ963680.1	3
HBS23508	JQ963681.1	3
HBS23509	JQ963682.1	3
HBS23510	JQ963683.1	8
HBS23511	JQ963684.1	3
HBS23512	JQ963685.1	8
HBS23513	JQ963686.1	3
HBS23517	JQ963687.1	3
HBS23533	JQ963688.1	3
HBS23534	JQ963689.1	3
HBS23536	JQ963690.1	3
HBS23540	JQ963691.1	9
HBS23545	JQ963692.1	3
HBS23548	JQ963693.1	3
HBS23590	JQ963694.1	3
HBS23599	JQ963695.1	3
HBS23602	JQ963696.1	3
HBS23610	JQ963697.1	3
HBS23616	JQ963698.1	10
HBS23625	JQ963699.1	3
HBS27866	JQ963827.1	3
HBS28515	JQ963857.1	3
HBS27370	JQ963789.1	3
HBS27373	JQ963791.1	3
HBS27760	JQ963825.1	3
HBS28273	JQ963853.1	3
HBS28277	JQ963854.1	1
HBS28296	JQ963855.1	1
HBS27748	JQ963821.1	3
HBS27752	JQ963822.1	3
HBS27753	JQ963823.1	3
HBS27754	JQ963824.1	3
HBS27770	JQ963826.1	3
HBS27044	JQ963768.1	3
HBS27045	JQ963769.1	3
HBS27047	JQ963770.1	3
HBS27048	JQ963771.1	3
HBS28668	JQ963867.1	3
HBS26328	JQ963754.1	11
HBS26330	JQ963755.1	11
HBS26917	JQ963759.1	11
HBS26918	JQ963760.1	11
HBS26919	JQ963761.1	11
HBS26920	JQ963762.1	11
HBS28625	JQ963864.1	13
HBS28644	JQ963865.1	12
HBS28654	JQ963866.1	12
HBS28177	JQ963848.1	3
HBS28185	JQ963849.1	3
HBS28205	JQ963850.1	3
HBS28256	JQ963851.1	3
HBS28263	JQ963852.1	3
HBS27429	JQ963793.1	6
HBS27448	JQ963794.1	1
HBS27461	JQ963795.1	1
HBS27466	JQ963796.1	1
HBS27476	JQ963797.1	5
HBS27486	JQ963798.1	1
HBS27495	JQ963799.1	1
HBS27502	JQ963800.1	1
HBS27513	JQ963801.1	1
HBS29249	JQ963868.1	1
HBS29255	JQ963869.1	1
HBS23630	JQ963700.1	3
HBS23652	JQ963702.1	3
HBS23654	JQ963703.1	3
HBS23670	JQ963706.1	3
HBS23674	JQ963708.1	3
HBS23675	JQ963709.1	3
HBS26939	JQ963767.1	3

Table B.4 Migration rates among the 25 sites.

The direction of migration is from the site below the diagonal to the site above the diagonal (e.g. migration from BE to BA is 0.02)

	BA	BE	BL	CV	DO	EL	ER	JO	KL	LO	NI	OF	PA	RE	RV	RO	RU	SI	SC	SH	SK	SW	TE	TI	WE
BA		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01
BE	0.02		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
BL	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
CV	0.01	0.01	0.01		0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.07	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01
DO	0.01	0.01	0.01	0.01		0.01	0.01	0.09	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01
EL	0.01	0.01	0.01	0.01	0.01		0.01	0.12	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
ER	0.01	0.01	0.02	0.01	0.03	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
JO	0.01	0.01	0.01	0.01	0.01	0.02	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
KL	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.14	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
LO	0.01	0.01	0.01	0.01	0.03	0.01	0.01	0.07	0.01		0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
NI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
OF	0.03	0.01	0.04	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.03	0.03	0.01	0.01	0.01	0.01
PA	0.01	0.01	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01
RE	0.05	0.01	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
RV	0.08	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01
RO	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
RU	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02
SI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01
SC	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01
SH	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01	0.01
SK	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01		0.01	0.01	0.01	0.01
SW	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01	0.01
TE	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01	0.01
TI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.15	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		0.01
WE	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	

B.2 Sites

Some of the sites where I sampled require further comment. Below are explanations of why I combined samples from separate water bodies into a single site for analysis and why some sites where samples were collected were excluded from the analyses in Chapter 2.

Baird and Hidden Lake

As indicated in Chapter 2, this site actually consists of two discrete water bodies: Baird Lake and Hidden Lake. These lakes are only 1.7 km apart as the bird flies, and there is a possibility of a stream connecting the two. In 2011 I sampled at Baird Lake but collected only seven samples. In 2012 I returned but did not observe many turtles and managed to catch only two more. Sampling at Hidden Lake in 2012 was more successful, resulting in the collection of 11 blood samples.

The finding in Chapter 2 that even nearby sites are demographically independent would seem to suggest that combining samples from Baird and Hidden Lakes into a single population for analysis is unjustified; however, it is highly doubtful that the major findings of my research would be altered by combining the sites for analysis purposes. No significant heterozygote deficit (i.e. a Wahlund effect, Wahlund 1928) was found to suggest that unrecognized population subdivision was present when the sites were combined.

Texada Island

As indicated in Chapter 2, this site actually consists of several discrete water bodies: Capsheaf Lake (n=3), Emily Lake (n=13), Priest Lake (n=1) and Case Lake (n=2). Considerations are similar as described for Baird and Hidden Lake above.

Miller Pond

This site near Clearwater (latitude 51.6554, longitude -120.0247) was sampled in 2012. The site consisted of a pond on private land that had a population of at least 150 western painted turtles. After collecting blood samples from 20 individuals we interviewed the land owner and were told that his family, which had lived on that property for four generations, had introduced turtles to that pond in the 1960's. He recalled that the turtles had originated from Stump Lake, near Kamloops, and had since spread from his property to nearby lakes, such as Dutch Lake in the town of Clearwater where western painted turtles had not previously been. Due to this anecdotal evidence that the turtles may have been introduced, this site was excluded from the analyses presented in Chapter 2. Preliminary site level analyses were performed, however, which did not indicate any of the genetic signatures typically associated with a recent founder event, calling into question the anecdotal evidence of a recent introduction.

Manly Oxbow

This site consists of a series of sloughs along a secondary road near Grand Forks (latitude 49.0067, longitude -118.3632). I sampled 20 individuals in 2012. Preliminary analyses indicated heterozygote deficit at this site, which is an indication that individuals that were introduced to that site were included in this sample; for this reason Manly Oxbow was excluded from the analyses in Chapter 2.

Bruning Creek

In 2012, Julie Steciw collected 12 road killed individuals from a 200 m stretch of Highway 97, near 150 Mile House (latitude 52.091504, longitude -121.920879). DNA was extracted from the tissue samples; however, it was of poor quality. I attempted to genotype the ten microsatellite loci for those individuals, but I had low confidence in the alleles called and the site was excluded from the analyses in Chapter 2.

Appendix C : Material supplemental to Chapter 3

C.1 Tables

Table C.1 Details about individuals used in each analysis

X or haplotype number, included in analysis; blank, not included in analysis

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
SI.01	*bellii*	BC	Cariboo	B.2		S.2				
SI.12	*bellii*	BC	Cariboo	B.2		S.3	S.2			
CV.01	*bellii*	BC	Kootenay	B.2		B.2	S.2			
CV.12	*bellii*	BC	Kootenay	B.2		S.3	S.2			
CV.17	*bellii*	BC	Kootenay	B.2		B.3		X		X
DO.01	*bellii*	BC	Kootenay	B.2		B.4				
DO.06	*bellii*	BC	Kootenay	B.2		B.4		X		X
EL.01	*bellii*	BC	Kootenay	S.1		S.2			X	
EL.12	*bellii*	BC	Kootenay	B.2		S.2				
ER.01	*bellii*	BC	Kootenay	B.2		B.2	S.2			
ER.06	*bellii*	BC	Kootenay	B.2		B.2		X		X
GR.01	*bellii*	BC	Kootenay	B.2		B.5	S.2			
GR.02	*bellii*	BC	Kootenay	B.2		S.2				
JO.01	*bellii*	BC	Kootenay	S.1		B.4				
JO.38	*bellii*	BC	Kootenay	S.1		S.2		X		X
LO.01	*bellii*	BC	Kootenay	B.2		B.4				
LO.12	*bellii*	BC	Kootenay	B.2		B.4				
LO.29	*bellii*	BC	Kootenay	B.2		S.2				

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
RO.01	*bellii*	BC	Kootenay	B.2						
RO.12	*bellii*	BC	Kootenay	B.3		S.2		X	X	X
RO.34	*bellii*	BC	Kootenay	B.3		B.3		X		X
NI.01	*bellii*	BC	Lower Mainland	B.2		S.2				
NI.29	*bellii*	BC	Lower Mainland	B.2		S.3	S.2			
NI.35	*bellii*	BC	Lower Mainland	B.2		S.2				
SL.37	*bellii*	BC	Lower Mainland	S.1		B.4		X		X
BA.01	*bellii*	BC	Okanagan	B.1		S.2		X	X	X
BA.10	*bellii*	BC	Okanagan	B.1		S.2		X		X
BA.801	*bellii*	BC	Okanagan	B.1		S.3	S.2			
BU.01	*bellii*	BC	Okanagan	B.2		S.3	S.2			
BU.10	*bellii*	BC	Okanagan	B.2		S.3	S.2			
BU.19	*bellii*	BC	Okanagan	B.2		S.2				
OF.27	*bellii*	BC	Okanagan	B.2		S.3	S.2			
OF.29	*bellii*	BC	Okanagan	B.2		S.3	S.2			
OF.30	*bellii*	BC	Okanagan	B.2		B.2				
RE.01	*bellii*	BC	Okanagan	B.2		B.2	S.2			
RE.03	*bellii*	BC	Okanagan	B.2		S.3	S.2			
RE.04	*bellii*	BC	Okanagan	B.2		S.2				
KL.34.01	*bellii*	BC	Sunshine Coast	B.2		S.2				
KL.34.12	*bellii*	BC	Sunshine Coast	B.2		B.2	S.2	X		X
KL.34.16	*bellii*	BC	Sunshine Coast	B.2		S.2				
TI.113	*bellii*	BC	Sunshine Coast	B.2		S.3	B.2			
TI.23	*bellii*	BC	Sunshine Coast	B.2						
TI.31	*bellii*	BC	Sunshine Coast	B.2		S.3	S.2			
WE.3137	*bellii*	BC	Sunshine Coast	B.2		S.2				
WE.318	*bellii*	BC	Sunshine Coast	B.2		B.2	S.2			
WE.34	*bellii*	BC	Sunshine Coast	B.2		B.2	S.2			

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
RV.32	*bellii*	BC	Thompson	B.2		S.2				
RV.85	*bellii*	BC	Thompson	B.1		S.2		X		X
RV.98	*bellii*	BC	Thompson	B.2		S.2				
SK.01	*bellii*	BC	Thompson	B.2		S.2				
SK.12	*bellii*	BC	Thompson	B.2		S.3	S.2			
SK.25	*bellii*	BC	Thompson	B.2		S.2				
BE.136	*bellii*	BC	Vancouver Island	B.2		S.2		X	X	X
BE.137	*bellii*	BC	Vancouver Island	B.2		S.3	S.2			
BE.17	*bellii*	BC	Vancouver Island	B.2		S.2		X		X
PA.24	*bellii*	BC	Vancouver Island	B.2		S.2				
PA.28	*bellii*	BC	Vancouver Island	B.2		S.2				
PA.36	*bellii*	BC	Vancouver Island	B.2		S.2				
HBS27532	*bellii*	CO	Douglas	S.1	JQ963802.1					
HBS27533	*bellii*	CO	Douglas	S.1	JQ963803.1	S.2	B.6	X		X
HBS28603	*bellii*	CO	La Plata	S.1	JQ963861.1					
HBS28610	*bellii*	CO	La Plata	B.13	JQ963862.1	B.3			X	
HBS28620	*bellii*	CO	La Plata	B.13	JQ963863.1	B.3		X		X
HBS28097	*bellii*	CO	Morgan	S.1	JQ963839.1					
HBS28101	*bellii*	CO	Morgan	S.1	JQ963840.1					
HBS28531	*bellii*	IA	Hamilton	B.12		B.6		X	X	X
HBS28535	*bellii*	IA	Hamilton	S.1	JQ963859.1					
HBS28303	*bellii*	ID	Boundary	B.2	JQ963856.1	S.2		X		X
HBS26298	*bellii*	IL	Carroll	S.1	JQ963751.1					
HBS26301	*bellii*	IL	Carroll	B.7	JQ963752.1				X	
HBS26322	*bellii*	IL	Carroll	S.4	JQ963753.1					
HBS28109	*bellii*	KS	Berton	B.11	JQ963841.1	S.2		X	X	X
HBS28111	*bellii*	KS	Berton	S.1	JQ963842.1	B.7	S.1	X		X
HBS28113	*bellii*	KS	Berton	S.1	JQ963843.1					

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS28134	*bellii*	KS	Sherman	S.1	JQ963844.1	S.2	B.6	X		X
HBS23534	*bellii*	MN	Atikin	S.1	JQ963689.1					
HBS23590	*bellii*	MN	Brown	S.1	JQ963694.1					
HBS23599	*bellii*	MN	Brown	S.1	JQ963695.1					
HBS23536	*bellii*	MN	Crow Wing	S.1	JQ963690.1					
HBS23540	*bellii*	MN	Crow Wing	B.5	JQ963691.1			X	X	X
HBS23616	*bellii*	MN	Houston	B.6	JQ963698.1				X	
HBS23625	*bellii*	MN	Houston	S.1	JQ963699.1					
HBS23517	*bellii*	MN	Itasca	S.1	JQ963687.1					
HBS23533	*bellii*	MN	Itasca	S.1	JQ963688.1					
HBS23545	*bellii*	MN	Nicollet	S.1	JQ963692.1					
HBS23548	*bellii*	MN	Nicollet	S.1	JQ963693.1					
HBS23602	*bellii*	MN	Nicollet	S.1	JQ963696.1					
HBS23610	*bellii*	MN	Nicollet	S.1	JQ963697.1					
HBS23506	*bellii*	MN	St Louis	S.1	JQ963679.1					
HBS23507	*bellii*	MN	St Louis	S.1	JQ963680.1					
HBS23508	*bellii*	MN	St Louis	S.1	JQ963681.1					
HBS23509	*bellii*	MN	St Louis	S.1	JQ963682.1					
HBS23510	*bellii*	MN	St Louis	B.4	JQ963683.1	B.6	B.9	X	X	X
HBS23511	*bellii*	MN	St Louis	S.1	JQ963684.1					
HBS23512	*bellii*	MN	St Louis	B.4	JQ963685.1					
HBS23513	*bellii*	MN	St Louis	S.1	JQ963686.1					
HBS27866	*bellii*	MN	Wabasha	S.1	JQ963827.1					
HBS28515	*bellii*	MN	Wabasha	S.1	JQ963857.1					
HBS28518	*bellii*	MN	Wabasha	S.4	JQ963858.1					
HBS27370	*bellii*	MO	Boone	S.1	JQ963789.1					
HBS27372	*bellii*	MO	Boone	B.7	JQ963790.1					
HBS27373	*bellii*	MO	Boone	S.1	JQ963791.1					

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS27376	*bellii*	MO	Boone	B.7	JQ963792.1	S.2	B.6	X		X
HBS28273	*bellii*	MT	Big Horn	S.1	JQ963853.1					
HBS28277	*bellii*	MT	Lewis and Clark	B.2	JQ963854.1	S.2		X		X
HBS28092	*bellii*	MT	Lincoln	S.1	JQ963838.1					
HBS28296	*bellii*	MT	Sanders	B.2	JQ963855.1					
HBS27760	*bellii*	MT	Sheridan	S.1	JQ963825.1					
HBS27770	*bellii*	ND	Kidder	S.1	JQ963826.1					
HBS27748	*bellii*	ND	Rolette	S.1	JQ963821.1	S.2	B.6	X		X
HBS27752	*bellii*	ND	Rolette	S.1	JQ963822.1					
HBS27753	*bellii*	ND	Rolette	S.1	JQ963823.1					
HBS27754	*bellii*	ND	Rolette	S.1	JQ963824.1					
HBS28668	*bellii*	NE	Cherry	S.1	JQ963867.1					
HBS27044	*bellii*	NE	Sand Hills	S.1	JQ963768.1					
HBS27045	*bellii*	NE	Sand Hills	S.1	JQ963769.1					
HBS27047	*bellii*	NE	Sand Hills	S.1	JQ963770.1					
HBS27048	*bellii*	NE	Sand Hills	S.1	JQ963771.1					
HBS28644	*bellii*	NM	Apache	B.15	JQ963865.1	B.1		X	X	X
HBS28654	*bellii*	NM	Apache	B.15	JQ963866.1					
HBS28625	*bellii*	NM	San Juan	B.14	JQ963864.1	S.3	B.2	X	X	X
HBS26917	*bellii*	NM	Sierra	B.8	JQ963759.1					
HBS26918	*bellii*	NM	Sierra	B.8	JQ963760.1					
HBS26919	*bellii*	NM	Sierra	B.8	JQ963761.1					
HBS26920	*bellii*	NM	Sierra	B.8	JQ963762.1					
HBS26328	*bellii*	NM	Socorro	B.8	JQ963754.1				X	
HBS26330	*bellii*	NM	Socorro	B.8	JQ963755.1					
HBS28177	*bellii*	SD	Bennet	S.1	JQ963848.1	S.2		X		X
HBS28185	*bellii*	SD	Bennet	S.1	JQ963849.1					
HBS28205	*bellii*	SD	Bennet	S.1	JQ963850.1					

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS28138	*bellii*	SD	Charles Mix	S.1	JQ963845.1					
HBS28146	*bellii*	SD	Charles Mix	S.1	JQ963846.1					
HBS28171	*bellii*	SD	Charles Mix	S.1	JQ963847.1					
HBS28256	*bellii*	SD	Meade	S.1	JQ963851.1					
HBS28263	*bellii*	SD	Meade	S.1	JQ963852.1					
HBS29249	*bellii*	WA	Clark	B.2	JQ963868.1	B.2				
HBS27461	*bellii*	WA	Grant	B.2	JQ963795.1	S.2				
HBS27466	*bellii*	WA	Grant	B.2	JQ963796.1					
HBS27476	*bellii*	WA	Grant	B.10	JQ963797.1				X	
HBS27495	*bellii*	WA	Okanogan	B.2	JQ963799.1	B.9	B.2	X		X
HBS27486	*bellii*	WA	Okawogan	B.2	JQ963798.1			X		X
HBS29255	*bellii*	WA	Skamawia	B.2	JQ963869.1					
HBS27502	*bellii*	WA	Spokane	B.2	JQ963800.1					
HBS27513	*bellii*	WA	Spokane	B.2	JQ963801.1	B.2				
HBS27429	*bellii*	WA	Yakima	B.9	JQ963793.1				X	
HBS27448	*bellii*	WA	Yakima	B.2	JQ963794.1					
HBS23630	*bellii*	WI	Chippewa	S.1	JQ963700.1					
HBS23635	*bellii*	WI	Chippewa	S.4	JQ963701.1				X	
HBS23652	*bellii*	WI	Chippewa	S.1	JQ963702.1					
HBS26245	*bellii*	WI	La Crosse	S.4	JQ963742.1					
HBS26939	*bellii*	WI	La Crosse	S.1	JQ963767.1					
HBS23656	*bellii*	WI	Lincoln		JQ963704.1			X		X
HBS27284	*dorsalis*	AL	Marshall		JQ963787.1	D.1		X		X
HBS23299	*dorsalis*	AR	Lonoke	D.2	JQ963670.1	S.1	D.2	X	X	X
HBS23319	*dorsalis*	AR	Lonoke	D.2	JQ963671.1					
HBS23324	*dorsalis*	AR	Lonoke	D.2	JQ963672.1					
HBS23169	*dorsalis*	IL	Alexander	D.1	JQ963656.1	D.3		X	X	X
HBS23176	*dorsalis*	IL	Alexander	D.6	JQ963663.1			X	X	X

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS26210	*dorsalis*	LA	Concordial	D.3	JQ963733.1			X	X	X
HBS26211	*dorsalis*	LA	Concordial	D.2	JQ963734.1					
HBS26212	*dorsalis*	LA	Concordial	D.4	JQ963735.1			X	X	X
HBS26921	*dorsalis*	LA	Concordial	D.2	JQ963763.1					
HBS27641	*dorsalis*	LA	Concordial	D.5	JQ963816.1				X	
HBS27651	*dorsalis*	LA	Concordial	D.1	JQ963817.1					
HBS31496	*dorsalis*	LA	Iberville	D.2	JQ963870.1					
HBS31533	*dorsalis*	LA	St Martin	D.2	JQ963872.1	S.6		X		X
HBS28542	*marginata*	IN	Boone	S.1	JQ963860.1				X	
HBS23366	*marginata*	IN	Kosciusko	S.4	JQ963673.1				X	
HBS23395	*marginata*	IN	Kosciusko	S.4	JQ963674.1	S.3		X		X
HBS31531	*marginata*	IN	Marion	S.4	JQ963871.1					
HBS27134	*marginata*	MI	Cheboygan	S.4	JQ963775.1					
HBS27135	*marginata*	MI	Cheboygan	S.4	JQ963776.1					
HBS27158	*marginata*	MI	Lenawee	S.4	JQ963780.1					
HBS27160	*marginata*	MI	Lenawee	M.11	JQ963781.1	S.3		X	X	X
HBS27161	*marginata*	MI	Lenawee	M.12	JQ963782.1				X	
HBS27141	*marginata*	MI	Mecosta	S.4	JQ963777.1					
HBS27147	*marginata*	MI	Mecosta	S.4	JQ963778.1					
HBS27151	*marginata*	MI	Mecosta	S.4	JQ963779.1					
HBS27109	*marginata*	MI	Schoolcraft	S.4	JQ963772.1					
HBS27117	*marginata*	MI	Schoolcraft	S.4	JQ963773.1					
HBS27124	*marginata*	MI	Schoolcraft	M.10	JQ963774.1			X	X	X
HBS26252	*marginata*	MI	Shiawassee	S.4	JQ963743.1					
HBS26256	*marginata*	MI	Shiawassee	S.4	JQ963744.1					
HBS26260	*marginata*	MI	Shiawassee	S.4	JQ963745.1					
HBS26261	*marginata*	MI	Shiawassee	S.4	JQ963746.1					
HBS26267	*marginata*	MI	Shiawassee	S.4	JQ963747.1					

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS26293	*marginata*	MI	Shiawassee	S.4	JQ963748.1					
HBS26353	*marginata*	MI	Shiawassee	M.9	JQ963756.1				X	
HBS28001	*marginata*	NY	Clinton	S.2	JQ963828.1			X		X
HBS26092	*marginata*	NY	Madison	M.4	JQ963726.1					
HBS26065	*marginata*	NY	Oneida	M.3	JQ963718.1				X	
HBS26068	*marginata*	NY	Oneida	M.4	JQ963719.1	S.4		X	X	X
HBS26069	*marginata*	NY	Oneida	M.2	JQ963720.1					
HBS26081	*marginata*	NY	Onondaga	M.2	JQ963721.1					
HBS26083	*marginata*	NY	Onondaga	M.2	JQ963722.1					
HBS26086	*marginata*	NY	Oswego	M.3	JQ963723.1					
HBS26090	*marginata*	NY	Oswego	M.5	JQ963724.1	S.8		X	X	X
HBS26091	*marginata*	NY	Oswego	M.2	JQ963725.1					
HBS26061	*marginata*	NY	Rochester	M.2	JQ963717.1	S.5	M.1	X	X	X
HBS27221	*marginata*	OH	Pike	M.14	JQ963785.1			X	X	X
HBS27227	*marginata*	OH	Pike	M.2	JQ963786.1					
HBS27179	*marginata*	OH	Shelby	S.4	JQ963783.1	M.2		X		X
HBS27185	*marginata*	OH	Shelby	M.13	JQ963784.1			X	X	X
Ont.3	*marginata*	Ont	Frontenac	M.1		M.3		X	X	X
Ont.4	*marginata*	Ont	Frontenac	S.2		M.4		X	X	X
Ont.5	*marginata*	Ont	Frontenac	S.2		S.5	M.3	X		X
Ont.6	*marginata*	Ont	Frontenac	S.2		M.5		X		X
Ont.7	*marginata*	Ont	Frontenac	S.2		M.1		X		X
Ont.8	*marginata*	Ont	Frontenac	S.2		M.5				
HBS26108	*marginata*	TN	Anderson	M.6	JQ963729.1				X	
HBS26112	*marginata*	TN	Anderson	M.7	JQ963730.1	S.5	S.3	X	X	X
HBS26113	*marginata*	TN	Anderson	M.8	JQ963731.1	S.7		X	X	X
HBS26114	*marginata*	TN	Anderson	M.7	JQ963732.1					
HBS28015	*marginata*	VT	Caledonia	M.15	JQ963829.1	S.8		X	X	X

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS28016	*marginata*	VT	Caledonia	S.3	JQ963830.1	M.6	M.4	X	X	X
HBS26213	*picta*	GA	Jackson	P.1	JQ963736.1				X	
HBS26214	*picta*	GA	Jackson	P.1	JQ963737.1	S.4		X		X
HBS26215	*picta*	GA	Jackson	P.1	JQ963738.1					
HBS26216	*picta*	GA	Jasper	P.1	JQ963739.1					
HBS26217	*picta*	GA	Jasper	P.1	JQ963740.1					
HBS26218	*picta*	GA	Jasper	P.1	JQ963741.1					
HBS26356	*picta*	GA	Jasper	P.1	JQ963757.1					
HBS27686	*picta*	GA	Macon	P.1	JQ963820.1					
HBS27654	*picta*	GA	Monroe	P.1	JQ963818.1	S.5		X		X
HBS27675	*picta*	GA	Monroe	P.3		P.1		X		X
HBS27676	*picta*	GA	Monroe	P.3	JQ963819.1				X	
HBS26038	*picta*	MA	Middlesex	P.4	JQ963713.1				X	
HBS26040	*picta*	MA	Middlesex	S.3	JQ963714.1					
HBS26042	*picta*	MA	Middlesex	P.5	JQ963715.1	S.6		X	X	X
HBS26058	*picta*	MA	Middlesex	S.2	JQ963716.1					
HBS33050	*picta*	MA	Worcester	S.2	JQ963877.1	S.2		X		X
HBS33053	*picta*	MA	Worcester	S.2	JQ963878.1					
HBS32992	*picta*	ME	Hancock	S.2	JQ963875.1					
HBS33030	*picta*	ME	Hancock	S.2	JQ963876.1					
HBS28035	*picta*	ME	Kennebec	S.2	JQ963833.1			X		X
HBS28041	*picta*	ME	Kennebec	S.2	JQ963834.1			X		X
HBS28044	*picta*	ME	Kennebec	S.2	JQ963835.1					
HBS28049	*picta*	NH	Salem	S.2	JQ963836.1	P.2	S.3	X		X
HBS28051	*picta*	NH	Salem	S.3	JQ963837.1					
HBS31658	*picta*	NJ	Sussex	S.2	JQ963873.1			X		X
HBS31678	*picta*	NJ	Sussex	S.2	JQ963874.1					
HBS27575	*picta*	NY	Sufflolk	S.3	JQ963804.1					

Voucher #	Putative ssp.	State	County	CR Haplotype	CR Accession #	PAX-P1 Haplotype 1	PAX-P1 Haplotype 2	Concatenated Tree	CR Tree	PAX Tree
HBS27577	*picta*	NY	Sufflolk	S.2		S.5		X		X
HBS27578	*picta*	NY	Sufflolk	S.3	JQ963805.1					
HBS27579	*picta*	NY	Sufflolk	S.2	JQ963806.1					
HBS27580	*picta*	NY	Sufflolk	P.6	JQ963807.1				X	
HBS27581	*picta*	NY	Sufflolk	S.2	JQ963808.1	S.6		X		X
HBS100115	*picta*	PA	Bucks			S.4		X		X
HBS26358	*picta*	RI	Kent	S.2	JQ963758.1			X	X	X
HBS26923	*picta*	RI	Kent	S.3	JQ963764.1				X	
HBS26924	*picta*	RI	Kent	S.2	JQ963765.1					
HBS26925	*picta*	RI	Kent	P.2	JQ963766.1	S.5		X	X	X
HBS27583	*picta*	VA	Fairfax	S.3	JQ963809.1	S.7	S.8	X		X
HBS27585	*picta*	VA	Fairfax	P.7	JQ963810.1			X	X	X
HBS27590	*picta*	VA	Fairfax	P.7	JQ963811.1					
HBS33304	*picta*	VA	Montgomery	S.3	JQ963879.1					
HBS23676		MI	Dickinson		JQ963710.1			X		X
HBS23690		MI	Dickinson		JQ963712.1			X		X
HBS28030		NH	Sullivan		JQ963832.1			X		X

Table C.2 Identity and source of outgroups

Taxon	Voucher ID	CR Accession #	CR Source	PAX Accession #	PAX Source
E. marmorata	HBS39753			GU085659	Barley et al. (2010)
E. marmorata	HBS39816	AY904894	Spinks and Shaffer (2005)		
P. megacephalum		DQ256377	Parham et al. (2006)		
P. megacephalum	HBS16255			GU085663.1	Spinks et al. (2013)
T. s .elegans	HSB23001		P.Q. Spinks, Personal Communication		P.Q. Spinks, Personal Communication
T. carolina	HSB27240	KC687248	Spinks et al. (2013)		P.Q. Spinks, Personal Communication
P. texana	RCT2	KC687245	Spinks et al. (2013)		P.Q. Spinks, Personal Communication, Tissue from Robert Thompson
G. geographica	RCT94		P.Q. Spinks, Personal Communication, Tissue from Robert Thompson		P.Q. Spinks, Personal Communication, Tissue from Robert Thompson