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Species delimitation in the *Choristoneura fumiferana* species complex (Lepidoptera: Tortricidae)

by

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For my parents,
Elmer & Margaret Lumley.
Thank you for your
unconditional love & support.

Abstract

Species identifications have been historically difficult in the economically important spruce budworm (*Choristoneura fumiferana*) pest complex. Morphological, ecological, behavioural, and genetic characters have been studied to try to understand the taxonomy of this group, but diagnostic character states differ in frequency rather than being complete replacements between each species. I developed a morphology-based character system that focuses on forewing colour components (Chapter 2), as well as eight simple sequence repeats (SSRs, also referred to as microsatellite markers) (Chapter 3). I tested these along with a 470 bp region of COI mitochondrial DNA (mtDNA) (Chapter 2, 4) to determine their congruence with putative species that were identified by adaptive traits (larval host plant, length of larval diapause, larval and adult morphology, pheromone attraction, distribution). The morphometrics system was effective for identification of the five species tested, with only slight overlap between *C. fumiferana* and *C. biennis*. MtDNA distinguished *C. fumiferana* and *C. pinus pinus*, but the remaining species shared haplotypes. SSRs distinguished four species (*C. fumiferana*, *C. pinus pinus*, *C. retiniana*, *C. lambertiana*) but the remaining four species that were included in this survey (Chapter 4) remained mixed within two populations. There was evidence for hybridization between several species pairs.

I also conducted a detailed study (Chapter 5) in Cypress Hills, an isolated remnant coniferous forest in western Canada, where identifying individuals from the *Choristoneura fumiferana* complex has been impossible due to the unusual

ecogeographic characteristics of the area. I integrated data on behaviour, ecology, morphology, mtDNA, and SSRs, comparing Cypress Hills populations to those from other regions of North America to determine which species they resembled most. I delimited at least three populations, resembling *C. fumiferana*, *C. occidentalis* and *C. lambertiana*. Adult flight phenology, along with pheromone attraction, were identified as major isolating mechanisms between these populations.

My studies highlighted the importance of integrative taxonomy for understanding species boundaries. Their patterns of differentiation suggest that spruce budworm species have recently diverged via natural selection in spite of some gene flow. Overall, this work is intended to contribute to more accurate identification of specimens and a better understanding of the evolutionary processes that drive speciation.

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COI: cytochrome *c* oxidase I

COII: cytochrome *c* oxidase II

MCMC: Markov-chain Monte Carlo

mtDNA: mitochondrial DNA

PCR: polymerase chain reaction

SSR(s): simple sequence repeats

Chapter 1

General Introduction

Species delimitation is the process by which distinct organismal entities are identified and delineated (Roe & Sperling, 2007). Morphology, life-history, behaviour, ecology, distribution, and genetic traits of species are all potential characters that can assist in their delimitation (e.g. Dayrat, 2005; Rissler & Apodaca, 2007; Padial *et al.*, 2009; Ross *et al.*, 2009). Accurate species delimitation and identification are important as they allow us a starting point to measure, monitor, manage, and conserve the Earth's biodiversity (Bickford *et al.*, 2006). However, accurately delineating species and finding appropriate characters for their identification are not always easy tasks, and are particularly difficult for closely related species in which the possibility for gene flow exists (Funk & Omland, 2003; Shaffer & Thomson, 2007). Species identification has historically been problematic for the closely related species within the spruce budworm (*Choristoneura fumiferana* Clemens) complex (Lepidoptera: Tortricidae) (Powell, 1980, 1995; Harvey, 1985; Nealis, 2008). The need for better delimitation techniques for species within this group stimulated the work comprised in this thesis.

The *Choristoneura fumiferana* complex

Life History

The spruce budworm species group is a pest complex that ranges across the coniferous regions of the Nearctic. *Choristoneura fumiferana* is Canada's most widespread, destructive, and economically devastating insect defoliator (Volney & Fleming, 2007), and has become a model organism for studying insect outbreak dynamics (e.g. Greenbank *et al.*, 1980; Royama, 1984; Williams & Liebhold, 2000; Royama *et al.*, 2005; Cooke *et al.*, 2007; Régnière & Nealis,

2007). Other species within the complex that have a tendency to enter an outbreak phase and cause extensive damage include *C. pinus pinus* Freeman, *C. occidentalis* Freeman, *C. biennis* Freeman, and *C. orae Freeman* (Nealis, 2008). Larvae feed on coniferous trees which can lead to extensive tree mortality during severe outbreaks, and significant reduction in growth rates even during moderate outbreaks (Nealis, 2008).

Budworms typically go through a one year life cycle, with the exceptions being *C. orae*, which can take one or two years (Harvey, 1967, 1985), and *C. biennis* which is fixed in a two year life cycle (Nealis, 2005). Generally, female adults lay up to 200 eggs in masses of 15-60 in a scale-like pattern on the undersides of needles, which hatch within two weeks (Nealis, 2008). Larvae do not feed, but spin silken hibernacula under bark scales, lichens, or within old staminate flower cups, then molt before overwintering as second instars (Nealis, 2008). They emerge the following year, often before bud flush, and then mine old needles or staminate flowers until eventually spinning silken webs in which to move and feed on the new needles, shoots, and flowers (Nealis, 2008). They tend to clip and leave uneaten needles, which turn reddish-brown and stick to the silken webbing along with their frass. This gives the trees a distinct reddish colouration during high spruce budworm densities, particularly at the crown where defoliation is typically the most extensive. Larvae go through five to eight instars (Nealis, 2008) and then pupate, emerging as adults approximately 10 days after pupation. Adults then fly for approximately two weeks. During this time, males are attracted to females by their sex pheromone; mating typically occurs within the tree crowns, and females then lay eggs (Nealis, 2008).

Known differences in life-history and behaviour among species include length and timing of stages within the life cycle (e.g. Smith, 1953, 1954; Volney *et al.*, 1983), daily activity patterns (e.g. Smith, 1953; Sanders, 1971a), larval diapause characteristics (e.g. Harvey, 1967; Nealis, 2005), larval host plant preference (e.g. Harvey, 1985), and pheromone chemistry (e.g. Sanders, 1971a,b; Silk & Kuenen, 1988; Powell & De Benedictis, 1995a). Species also differ in geographical distribution and morphology (e.g. MacKay, 1953, 1962; Freeman,

1967; Harvey & Stehr, 1967; Stehr, 1967; Dang, 1985, 1992; Harvey, 1985; Powell & De Benedictis, 1995b; Shepherd *et al.*, 1995).

Taxonomic History

The genus *Choristoneura* was proposed by Lederer in 1859, with *Tortrix diversana* Hübner as the type species. According to Brown *et al.* (2005) this genus includes thirty-eight species. Seventeen were described from the Nearctic Region, twenty from the Palearctic Region, and one from the Afrotropical Region (Wang & Yang, 2008). On the basis of male genitalic characters, Dang (1992) divided the genus into nine species groups. One group was the Nearctic conifer-feeders which was restricted to species from the *Choristoneura fumiferana* complex. This group was deemed to be more closely related to the Palearctic conifer-feeding group (*C. diversana*, *C. murinana*, *C. metasequoiacola*, *C. jezoensis*) than to other groups within the genus.

The complicated taxonomic history of the spruce budworm complex is recounted in detail by Powell (1995). In summary, *Choristoneura fumiferana* was the first species within the complex to be described, based on specimens collected in Virginia, and was originally placed in the genus *Tortrix* by Clemens in 1865. Soon after, other conifer-feeding moths were named, including *retiniana* Walsingham, 1879, *lambertiana* Busck, 1915, and *carnana* Busck and Barnes, 1920. McDunnough (1939) reassigned many *Tortrix* species to *Archips* (=*Cacoecia*) based on the tortricid classification proposed by Pierce and Metcalfe (1922). This brought all of the described budworms together into one genus, *Archips*.

Freeman (1947) transferred *fumiferana* to the genus *Choristoneura*. During this period, more detailed taxonomic studies were being undertaken on the pine-feeding budworm in eastern Canada (Brown & MacKay, 1943; Campbell, 1953; Cox, 1953; Freeman, 1953; MacKay, 1953; Smith, 1953) which culminated in *Choristoneura pinus* Freeman being named in 1953. Studies continued on the group, with a set of papers being published in 1967 to formalize the species and subspecies status of *C. occidentalis* Freeman, *C. biennis* Freeman, *C. orae*

Freeman, and *C. pinus maritima* Freeman, (Campbell, 1967; Freeman, 1967; Freeman & Stehr, 1967; Harvey, 1967; Harvey & Stehr, 1967; Stehr, 1967). At this time, Obstratov (1962) and Powell (1964) were focusing on species in the western United States, which culminated in several subspecies being named: *C. lambertiana ponderosana* Obraztsov, *C. lambertiana subretiniana* Obraztsov, and *C. carnana californica* Powell. Throughout this period, there were several taxonomic mix-ups as well as changes in species assignment or status that added to the problems of resolving this group (Powell, 1995).

Powell and De Benedictis (1995b) continued their taxonomic work in the western United States where they reaffirmed the designation of several previously described species (*C. carnana carnana, C. carnana californica, C. occidentalis, C. lambertiana lambertiana, C. lambertiana subretiniana, C. lambertiana ponderosana),* plus gave two species new status (*C. retiniana retiniana* (Walsingham), *C. retiniana spaldingiana* Obraztsov).

Overall, Brown *et al.* (2005) recognizes 12 species and subspecies in the spruce budworm complex (*C. biennis, C. carnana carnana, C. carnana californica, C. fumiferana, C. lambertiana lambertiana, C. lambertiana ponderosana, C. lambertiana subretiniana, C. occidentalis, C. orae, C. pinus pinus, C. pinus maritima, C. retiniana).* Powell and De Benedictis (1995b) recognize one additional subspecies (*C. retiniana spaldingiana*).

Taxonomic Dilemmas

Many studies have focused on the spruce budworm complex with the intent of finding consistent identifying characters for the species. Species within the spruce budworm complex are mainly distinguished by ecological and behavioural traits such as larval host plant, pheromone attraction, and length of larval diapause along with differences in larval and adult morphology (Freeman, 1967; Harvey, 1985). However, several kinds of evidence are typically needed to identify specimens because the defining characters vary in frequency among species rather than being complete character substitutions (Harvey, 1985, 1997; Dang, 1992). Currently, the most reliable method for species identification is to

consider first the larval host plants and geographical distribution of the specimens of interest (Nealis, 2008). Although locality information is typically available for collected specimens, larval host plant is generally unavailable for specimens collected as adults. Along with these challenges, all species are known to hybridize freely and produce viable offspring in the laboratory (Harvey, 1997). Hybrids have also been identified in nature (e.g. Volney *et al.*, 1984; Powell & De Benedictis, 1995a), but the extent of natural hybridization is unknown. Consequently, more reliable and consistent methods for species identification are needed to help monitor and manage this economically important pest complex. Determining appropriate methods for species delimitation within the spruce budworm complex may help focus taxonomic efforts for other closely related species. By studying the extent of gene flow among spruce budworm species, along with their differing behavioural, life-history, and morphological traits, it may also be possible to determine the mechanisms by which they maintain their genomic integrity.

Thesis overview

Finding fixed characters for identification, or finding morphological or molecular identifiers that reduce the need for detailed ecological data, would greatly help in species identification. This has led to the work described in Chapters 2, 3 and 4. For Chapter 2, I developed and tested 47 forewing morphometric characters, based on forewing measurements, colouration, and pattern, in order to determine their usefulness compared to mitochondrial DNA (mtDNA) for delimiting the five described *Choristoneura* species that reside in Alberta and south-eastern British Columbia. For Chapter 3, I developed simple sequence repeats (SSRs, also referred to as microsatellite markers) from *C. fumiferana* and *C. occidentalis*, and tested their ability to cross-amplify in the remaining species within the complex as well as in other species within the genus. For Chapter 4, I determined the usefulness of mtDNA and SSRs for delimiting all

species in the *Choristoneura fumiferana* complex in North America, sampled from across their known geographical range.

Since spruce budworm identification is reliant on knowing the ecogeographical characteristics of the region in which the specimens were sampled, it can be difficult, if not impossible, to identify individuals to species if they are collected outside of their known range. This difficulty was faced in the identification of individuals to species from Cypress Hills, a forested island situated hundreds of kilometres away from two major ecosystems (boreal and cordilleran), that hosts a combination of ecogeographical traits from both of these regions. In Chapter 5, I assigned Cypress Hills specimens to species units by using a combination of life history traits, morphology, mtDNA, and SSRs, and through a comparison of Cypress Hills specimens to the North American collection (Chapter 4). I also identified some intermediate genetic phenotypes that may indicate possible hybridization events, and identified differences in adult flight phenology as the possible mechanism for the maintenance of sympatric species units.

Overall, this work was intended to provide methods for delimiting species from the *Choristoneura fumiferana* complex, to explore the extent of gene flow or hybridization among species, and to determine possible reasons for why or how these species are maintaining their genomic integrity when they have the ability to hybridize and produce viable offspring.

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Chapter 2

Integrating morphology and mitochondrial DNA for species delimitation within the spruce budworm (*Choristoneura fumiferana*) cryptic species complex (Lepidoptera: Tortricidae)

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Introduction

Cryptic species complexes are defined as assemblages of closely related species that have been or are now sometimes classified as one broadly delimited species due to the difficulty in identifying them on the basis of visible phenotype (Collins & Paskewitz, 1996; Bickford *et al.*, 2006). Such species complexes, by definition, present a worst case challenge for the use of morphological characters in species delimitation and identification.

The development of DNA-based taxonomy over the past several decades (e.g. Ayala & Powell, 1972; Berlocher, 1980) has largely assisted in diagnosing species that are difficult to distinguish morphologically. Insects are an especially appropriate subject for molecular taxonomy, as they make up such a large component of the Earth's biodiversity and their small size and morphological similarity make them difficult to identify using traditional taxonomic characters (Berlocher, 1984; Caterino *et al.*, 2000; Sperling, 2003; Sperling & Roe, 2009). DNA barcoding (Hebert *et al.*, 2003a, b), in which a 658 bp region of mitochondrial cytochrome *c* oxidase I (COI) has been proposed as a standard sequence for the identification of animals, has provided evidence for the utility of this region of mitochondrial DNA (mtDNA) for the discovery or confirmation of cryptic species (e.g. Hebert *et al.*, 2004). Further, some supporters of DNA

barcoding (Packer *et al.*, 2009) have recently discussed the "mediocrity of morphology", maintaining that DNA barcoding "nearly always outperforms morphology" in cryptic species recognition. However, because species complexes are also closely related, a single DNA sequence region may not suffice for identification either, as these species may still have the ability to hybridize and exchange genes (Funk & Omland, 2003). Alternatively, species can contain multiple divergent DNA lineages or, for that matter, polymorphic characters of any kind, where the extent of intraspecific variation is as high or higher than interspecific variation (e.g. Cognato, 2006; Meier *et al.*, 2006). Variation in divergence rates between gene regions may introduce stochastic error into identifications (Roe & Sperling, 2007). DNA introgression between species, retained ancestral polymorphism, and stochastic variation can all result in biologically valid, low divergence between species in mtDNA or any other single DNA region. In this case, other genetically determined characters (ecological, behavioural, morphological, or molecular) may delineate such species more effectively.

My objective for this study was to determine the most appropriate method to identify and delimit species in the spruce budworm (*Choristoneura fumiferana* Clemens, 1865) complex, a group of species that has already been acknowledged widely as an unresolved challenge for morphology-based identification (Powell, 1995). Preliminary studies of this complex by Sperling & Hickey (1994, 1995) obtained promising results from mtDNA and they recommended its use for species identification. I employed a multivariate approach using wing pattern characters to determine whether better resolution can be obtained with morphology or mtDNA, or if integration of the two methods is necessary to delimit the species. I challenged the value of these methods by focusing on a geographical region that has the highest number of potentially interacting spruce budworm species in North America.

The spruce budworm (*Choristoneura fumiferana*) species group is a pest complex that feeds on coniferous trees across the Nearctic, with *C. fumiferana* being the continents' most destructive insect defoliator (Volney and Fleming,

2007). Currently thirteen species and subspecies are recognized formally in the complex (Freeman, 1967; Powell, 1995) and a total of 15 'biotypes' have been identified recently (Volney and Fleming, 2007). Species are distinguished by larval host plant preference (Stehr, 1967), geographical range (Stehr, 1967), length of larval diapause (Harvey, 1967), larval and pupal morphology (MacKay, 1953, 1962; Harvey & Stehr, 1967), and adult morphology (Freeman, 1967). However, these species all qualify as cryptic because they lack complete character substitutions among them, with defining characters differing only in frequency between species (Harvey, 1985; Dang, 1992; Harvey, 1997). Identification of larval host plant is the easiest method to determine the species, but this information is not available for individuals collected as adults. Since the species descriptions of Freeman (1967), many studies have attempted to resolve the taxonomy of the spruce budworm complex using characters from genitalia (Dang, 1985, 1992; De Benedictis, 1995), eggs (Harvey, 1983, 1997), larval, pupal and adult colour (Volney *et al.*, 1983, 1984), adult forewing and hindleg basitarsal spine length (De Benedictis, 1995), pheromone attraction (e.g. Sanders, 1971; Sanders *et al.*, 1977; Silk & Kuenen, 1988; Powell, 1995), larval host association (Volney *et al.*, 1984; Powell & De Benedictis, 1995), sex chromatin and chromosome numbers (Ennis, 1976), allozymes (Castrovillo, 1982; Harvey, 1996) and mtDNA (Sperling & Hickey, 1994, 1995). The most recent bibliography of *C. fumiferana*, although published over 20 years ago (McKnight *et al.*, 1988), cited 4318 reports and papers mentioning some aspect of the biology of the spruce budworm species complex. Clearly, effective species identification for this group would serve a diversity of forestry and basic research needs, and help to focus taxonomic efforts for other such complexes.

Materials and Methods

Collections

Specimens of the *C. fumiferana* complex were sampled during 2005 to 2008 in Alberta and south-eastern British Columbia by collecting larvae from

their host trees and rearing them to the adult stage. For one species, *C. pinus pinus* Freeman, 1953, some adults were collected using pheromone traps baited with lures, as described by Silk *et al.* (1985). For larval collections, the tree species upon which larvae fed, larval head colouration, and larval diapause characteristics were recorded. Larvae were reared to the adult stage on the plant foliage from which they were collected. Larvae that went into second diapause during the fourth instar were placed at 2^{o}C for approximately 6 months before rearing was continued. Adults were stored at -70^{o}C.

I limited this study to specimens associated with sufficient ecological, behavioural, and larval morphological data to unambiguously identify them to species based on the original descriptions (Freeman, 1967) or taxonomic reviews (Harvey, 1985; Powell, 1995). All species previously recorded from Alberta and south-eastern British Columbia were sampled, including *C. fumiferana* (n = 38), *C. p. pinus* (n = 32), *C. occidentalis* Freeman, 1967 (n = 17), *C. biennis* Freeman, 1967 (n = 16), and *C. lambertiana* Busck, 1915 (n = 8) for a total of 111 individuals (Figure 2-1, Table 2-1). *Choristoneura fumiferana* larvae were collected on white spruce [*Picea glauca* (Moench) Voss] and had dark brown to black heads. *Choristoneura pinus pinus* larvae were collected on jack pine (*Pinus banksiana* Lamb.) and had reddish-brown heads with dark brown to black prothoracic shields. Very few *C. p. pinus* larvae were located, so pheromone trap material was also included from localities where only one *Choristoneura* host plant, *Pinus banksiana*, was available for larval feeding. *Choristoneura pinus pinus* pheromone lures consisted of a 9:1 ratio of 85:15 (*E*,*Z*)-11-tetradecenyl acetates and 85:15 (*E*,*Z*)-11-tetradecen-1-ols (Silk *et al.*, 1985) and were obtained from the Canadian Forest Service. *Choristoneura occidentalis* larvae were collected on Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] and had light reddish-brown heads and prothoracic shields. *Choristoneura biennis* larvae were collected from Engelmann spruce (*Picea engelmannii* Parry ex Engelmann) in high elevation stands, had light reddish-brown heads with dark brown to black lateral stripes, and went through a second diapause. *Choristoneura lambertiana* larvae were collected on lodgepole pine (*Pinus contorta* Douglas ex Louden) and

had light reddish-brown heads with darker brown to black prothoracic shields. Prothoracic shield colouration was not recorded for *C. fumiferana* and *C. biennis* as it was unnecessary for identification at the time of collection. Voucher specimens and images were deposited in the E. H. Strickland Entomological Museum at the University of Alberta. Three outgroup species were used to root the molecular analysis, including one specimen each of *C. rosaceana* Harris, 1841 (Parry Sound, Ontario), *C. conflictana* Walker, 1863 (Little Spring, Arizona), and *C. murinana* Hübner, 1799 (Alsace, France). The outgroup specimens were chosen on the basis of being from the same genus but not part of the coniferophagous *C. fumiferana* species group and because mtDNA sequence was already available (Sperling & Hickey, 1994; unpublished). *Choristoneura rosaceana* and *C. conflictana* typically feed on deciduous trees or shrubs in the Nearctic region, and *C. murinana* is a conifer feeder in the Palearctic region (Dang, 1992).

Morphometric Measurements

Moths were pinned and spread to dry on flat plastazote boards (Landry & Landry, 1994). They were photographed in natural lighting with an 8 megapixel Canon EOS Digital Rebel XT camera and 272EE Tamron SP AF90mm F/2.8 Di Macro 1:1 lens. To maximize photograph coverage, but also to include the entire specimen in the photograph, a large-sized individual was used to determine the minimum length between specimens and the end of the in-focus camera lens. On the basis of this exercise, all moths were positioned 17.7 cm from the end of the in-focus camera lens, with wings flat and parallel to the lens. Manual focus was used for minor corrections and to remove automatic focal length changes by the camera. Custom white balance was checked and standardized regularly using the same sheet of white paper to retain consistent colour balance.

Twenty-five morphometric wing pattern elements were examined based on characters that have been used to distinguish the species in previous taxonomic publications (Freeman, 1967; Harvey, 1985; Powell, 1995), together with my own observations (Table 2-2, Figure 2-2). Photographs were imported into ImageJ

1.38x (Rasband, 2006) and this software was used for all morphometric measurements. If both the left and right wing were in equal condition then the right wing was used. Worn specimens and specimens slightly discoloured from oils permeating the wings during the pinning and drying process were included in the analysis. We included these specimens to determine the practicality of the method, as spruce budworm adults are often caught using pheromone or blacklight traps and are not in perfect condition. For colour measurements, the area being analysed was outlined, a colour histogram was generated and the mean proportion of red, blue, and green for each area was recorded. Therefore, measurements of colour yielded three numbers for each of 11 wing pattern elements, for a total of 33 morphometric characters. For an additional 14 morphometric characters, length, width and area were measured by drawing a line across or around the region of interest and recording the generated value. For the number of dark scales within the entire forewing (number 12 in Table 2-1; Figure 2-2), the picture was changed from colour to grayscale (8-bit). The image of the entire wing being measured was then highlighted, copied and pasted into a new screen. The image threshold was set between 0 and 23 and the area of black was recorded in pixels.

All morphometric measurements were transformed by $\log_{10}(x+1)$ and analysed by linear discriminant analysis in Ginkgo v1.4 (De Cáceres *et al.*, 2003) as well as in Systat v12 (Wilkinson, 1990), with species identification as per larval characteristics or adult pheromone attraction being the prior method of grouping individuals. Canonical discriminant functions were generated with training set resubstitution evaluation and leave-one-out evaluation to determine the replicability and reliability of the classification system. Linear discriminant analysis with backward stepwise selection helped to determine if any variables could be removed from the analysis.

Molecular Methods and Analysis

Substantial taxonomic variation in divergence rates has been demonstrated between different regions of the COI gene of mtDNA (Roe and Sperling, 2007),

so I compared full-length sequences of this gene within *Choristoneura* to determine which region would provide the most cost-effective information for this study. In particular, the full 2300 bp region of COI and COII mtDNA, the 658 bp DNA barcode region of COI, and the adjacent 470 bp region of COI mtDNA used previously by Sperling and Hickey (1994) were compared for five previously sequenced ingroup individuals, plus 2 outgroup specimens (Table 2-3). To allow analyses comparable with DNA barcoding studies (Hebert *et al.*, 2003a), the Kimura 2-parameter method was used to calculate corrected pairwise distances, which then provided percentage sequence divergences and neighbour-joining trees. The tree topologies were identical for all three regions. The average percent sequence divergence between all pairwise combinations of the ingroup individuals was 1.62% for the 2300 bp region, 1.11% for the 658 bp barcode region and 2.55% for the 470 bp region used by Sperling and Hickey (1994). Because this showed that the 470 bp region had a typical topology, yet a greater total number of potentially informative mutations than the barcode region, I continued to use the more informative 470 bp region of COI for this study.

Genomic DNA was extracted from leg and thorax tissue using QIAamp DNA minikits (Qiagen, Canada). Two different primer pairs were used for polymerase chain reaction (PCR) amplification to target the 470 bp region of COI mtDNA. Specimens collected in 2005 were amplified with previously published primers Jerry (C1-J-2183 5'-CAACATTTATTTTGATTTTTTGG-3') and Mila (C1-N-2659 5'-GCTAATCCAGTGAATAATGG) (Simon *et al.*, 1994). Specimens collected after 2005 were amplified with newly designed primers Bert (C1-J-2136 5'-CACATCATTTTTCGATCCT-3') and Ernie (C1-N-2668 5'-AGGGTTTAAAGCTAATCCAGT-3'). PCR reactions were conducted in 50 µL reactions containing 1 µL genomic DNA, 5 µL 10x PCR buffer containing 15 mmol/µL $MgCl_2$ (Promega, Madison, WI), 5 µL of 25 mmoles/µL $MgCl_2$ (Promega), 1 µL of 10 mmoles/µL dNTPs (Roche, Switzerland), 1 µL each of the forward and reverse primers in 5 pmol/µL concentrations, 0.5 µL of 5 U/µL *Taq* polymerase (Pickard laboratory, University of Alberta) and 35.5 µL autoclaved Millipore water. PCR amplification was conducted using the following

conditions: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 2 min, then 72 °C for 5 min. The PCR product was purified using either a QIAquick PCR purification kit (Qiagen) or ExoSAP-IT (USB Corporation, Cleveland, OH), then sequenced using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequence was purified by Sephadex G-50 fine columns (Amersham Biosciences Inc., Piscataway, NJ) or ethanol precipitation. Sequencing reactions were run on an ABI Prism 3730 DNA analyser and then assembled, edited, and visually checked in Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned by eye in PAUP 4.0b10 (Swofford, 2003). Thirteen unique new COI sequences were deposited in GenBank (accession numbers GQ890278 - GQ890295), and other sequences were identical to those published previously by Sperling and Hickey (1994) and Roe and Sperling (2007) (Table 2-3).

Nucleotide diversity (Tajima, 1983) was calculated using Arlequin v3.1 (Excoffier *et al.*, 2005). Intra- and interspecific sequence divergences and neighbour-joining trees were calculated using the Kimura 2-parameter-corrected distances. Maximum parsimony analysis using PAUP 4.0b10 (Swofford, 2003) was completed for haplotypes using heuristic search, random sequence addition, tree-bisection-reconnection and branch swapping with 1000 replications. Branch support was estimated using bootstrapping with 1000 replications using PAUP 4.0b10. A Bayesian analysis was carried out with MrBayes 3.1 (Ronquist & Huelsenbeck, 2003) using the general time reversible model of substitution with gamma-distributed rate variation across sites and a proportion of invariant sites (GTR+G+I). The Markov chain Monte Carlo calculation was allowed to run for 1,000,000 generations and trees were sampled every 100 generations. The first 25% of trees were discarded as burnin and the remaining trees were summarized into a phylogram with mean branch lengths and viewed in Treeview (Page, 1996). TCS v1.21 (Clement *et al.*, 2000) was used to generate a haplotype network using statistical parsimony (Templeton *et al.*, 1992). To allow a more direct comparison to the morphometric analysis, a linear discriminant analysis was performed in Systat v12 (Wilkinson, 1990) for all variable sites within the 470 bp sequence,

with each of the four nucleotides being characterized as a different category. Species identification was the prior method for grouping individuals as indicated by larval characters or adult pheromone attraction. An analysis was performed under no selection as well as backward stepwise selection. Morphological and mtDNA characters were also combined to determine if this would increase the accuracy of species determination.

Results

Morphometrics Analysis

The range of morphometric character values for each species is shown in Table 2-4. The first and second canonical axes of the linear discriminant analysis (Figure 2-3) grouped together individuals within each species and separated species from each other. There was no overlap except for *C. fumiferana* and *C. biennis*, which are not sympatric in nature. The overlap between these two species was due to one specimen of *C. biennis* and two specimens of *C. fumiferana*, out of a total of 54 specimens analysed for these species. Two of these specimens were worn and the third was partially discoloured due to oils permeating the wing during the pinning and drying process. Also, one of the *C. fumiferana* specimens was a brown female, which is a relatively rare, sex-limited colour trait for this species (Stehr, 1955). *Choristoneura pinus* remained separate on the plot of the first against the third canonical axis, but with more overlap between the other four species. *Choristoneura lambertiana* formed a distinct cluster in the plot of the second and third axis (not shown), with more overlap among the remaining species. Thus, the linear discriminant analysis of wing traits separated all species well, except for *C. biennis* and *C. fumiferana,* which are typically allopatric in nature.

The first four canonical discriminant functions explained 100% of the morphological variation among the five species, with Wilks' lambda test of functions being significant ($P<0.05$). Under no selection, LD1, LD2 and LD3 explained 63.8, 17.7 and 12.5% of the variation, respectively. Values of the

canonical discriminant functions are given in Table 2-5. Training set resubstitution evaluation matched 99% of individuals to the correct species whereas leave-one-out evaluation matched 77% of individuals to the correct species (Table 2-7). Specimens not correctly classified were mainly *C. fumiferana* misidentified as *C. biennis* and *C. biennis* misidentified as *C. fumiferana*.

Backward stepwise selection indicated that 16 variables could be used to separate taxa effectively. Canonical discriminant functions for these variables are given in Table 2-6. However, using only these 16 variables reduced species resolution noticeably on the LD axes. Training set resubstitution evaluation reduced matches to 93%, but leave-one-out evaluation increased to 85% in comparison with using all variables (Table 2-7).

Overall, colour characters were given the highest value for discriminating among species across the first three canonical axes. Based on the linear discriminant analysis of all 47 characters (Table 2-5), the characters that best discriminate among species on the first canonical axis are the colour of the entire forewing (red, blue, and green means) and the green colour mean of the costal spot. On the second canonical axis, the green colour mean of the subapical patch, entire forewing, costal spot, subterminal spots and median band were the most important characters. For the third canonical axis, the green and red colour means of the entire forewing, and the green colour mean of the costal spot and subterminal spots are the most highly scored characters for discrimination among species. Under backward stepwise selection (Table 2-6), the most discriminating characters along the first canonical axis are the green colour means of the costal spot, subterminal spots, and subapical patch along the costal margin. Along the second axis, they are the red, green and blue colour means of the costal spot. The most informative characters along the third canonical axis are the green colour means of the subapical patch along the costal margin and the subterminal spots.

Molecular Analysis

In total, 19 ingroup haplotypes were obtained from the analysis of 111 ingroup individuals sequenced over the 470 bp region of COI and were named in

accordance with Sperling and Hickey (1994) as per Table 2-3. Of the 470 base pairs, 416 remained constant, 22 were variable but parsimony uninformative, and 32 were parsimony informative. Forty-four character changes were unambiguous, as shown in Figure 2-4. Intraspecific sequence divergence, derived from corrected (Kimura 2-parameter) genetic pairwise distances, ranged from 0 to 0.86% whereas interspecific variation ranged from 0 to 4.46% (Figure 2-5). Under the two or three percent sequence divergence criteria used in DNA barcoding to delimit species (Hebert, 2003a), *C. fumiferana* is the only species with sufficiently high divergence to be considered a separate species. The remaining four taxa would be amalgamated into one species.

The Bayesian analysis was used as the basis for Figure 2-4. Neighbour-joining and maximum parsimony yielded very similar topologies and therefore their bootstrap values were added to the tree in Figure 2-4. *Choristoneura fumiferana* was recovered as a distinct and well-supported clade. *Choristoneura pinus* haplotypes were unique to *C. pinus* and constituted a paraphyletic sister group to the haplotypes from the western species. The western species *C. occidentalis*, *C. biennis* and *C. lambertiana* shared haplotypes within the remaining clade.

The haplotype network (Figure 2-6) resulted in a similar topology, with *C. fumiferana* sufficiently distinct that it was separated from the remaining network. The other haplotypes were joined, again with *C. pinus* forming a distinct group and the remaining species (*C. occidentalis*, *C. biennis*, *C. lambertiana*) sharing haplotypes within a third cluster.

When mtDNA was analysed using linear discriminant analysis, *C. pinus* and *C. fumiferana* formed distinct clusters. The remaining three sympatric species, *C. occidentalis*, *C. biennis*, and *C. lambertiana,* did not form unique clusters as they were overlapping. The first four canonical discriminant functions explained 100% of the variation and Wilks' lambda test of functions was significant ($P<0.05$). Under no selection, the LD1, LD2, and LD3 axes explained 83.3, 15.9, and 0.5% of the variation, respectively. Overall, under both no selection and backward stepwise selection, training set resubstitution evaluation

matched 89% of individuals to the correct species and leave-one-out evaluation matched 83% of individuals to the correct species (Table 2-7).

Combined Character Analysis

When morphology and mtDNA were combined and analysed using linear discriminant analysis, *C. fumiferana*, *C. pinus*, and *C. lambertiana* formed distinct clusters with space between them. *Choristoneura occidentalis* and *C. biennis* formed distinct clusters, but with no gap between. The first four canonical discriminant functions explained 100% of the variation and Wilks' lambda test of functions was significant ($P<0.05$). Under no selection, the LD1, LD2, and LD3 axes explained 70.5, 25.4 and 2.6% of the variation, respectively. Under both no selection and backward stepwise selection, training set resubstitution evaluation matched 100% of individuals to the correct species and leave-one-out evaluation matched 90% of individuals to the correct species (Table 2-7). Overall, combining morphology and mtDNA character sets gave the most successful results for reclassifying individuals to species using both the resubstitution and leave-one-out evaluation methods.

Discussion

Using morphometric analysis of wing characters, three of the five *Choristoneura* species included in this study form unique clusters. The remaining two species (*C. fumiferana* and *C. biennis*) are not completely distinguished, but these two species have geographical ranges that are mainly allopatric. In contrast, mtDNA separates two of the five taxa, with the remaining three western species sharing haplotypes. Mitochondrial DNA used in conjunction with morphometrics clearly helps to identify some species pairs, especially *C. fumiferana* and *C. biennis*, in which worn or discoloured specimens are less likely to be discerned using wing character morphometric methods. On the basis of this study, mtDNA or morphometrics could be used alone in species determination of the boreal species, *C. fumiferana* and *C. pinus*. Morphometrics should be included in species

determination within the cordilleran region to distinguish between *C. occidentalis*, *C. biennis*, and *C. lambertiana*, and combining character sets in this region will further increase the success of identifying individuals to species. Combining character sets would also be appropriate along the transition zone, between the boreal and cordilleran regions, where all five species can potentially interact.

Once familiarity is gained with the morphometric method described here for species identification, this method requires approximately 20 min per specimen to photograph and score all characters. There is also strong potential for the development of more computationally sophisticated visual recognition methods (LaSalle *et al.*, 2009) using artificial neural networks, such as Digital Automated Identification System (DAISY) (Gaston & O'Neill, 2004; Watson *et al.*, 2004) or machine learning techniques, such as Waikato Environment for Knowledge Analysis (WEKA) (Witten and Frank, 2005; Mayo & Watson, 2007). Sequencing mtDNA typically takes several hours, but can be done in 90 min given appropriate equipment and set-up (Consortium for the Barcode of Life, 2008).

Colour was of particular importance in delimiting spruce budworm species using the morphometrics-based method. The original description of the species group (Freeman, 1967) also relied heavily on colour for identifying adult specimens. Two problems identified with the traditional use of colour are that hues intergrade in such a way that it is difficult, if not impossible, to separate them into discrete units, and that it is difficult to consistently communicate the identity of a colour. As one example, Freeman (1967) included the colours reddish, more reddish, reddish-brown, reddish ochreous, ochreous and rust-coloured in the species description for the *C. fumiferana* complex, yet it may be difficult to interpret the true differences between these colours by a reader lacking opportunity to examine type material. Quantifying colour limits these issues and also reduces subjectivity and human error. The method for quantifying colour developed in this study could be applicable not only for cryptic species complexes but for a range of taxonomic studies, as colour is often used in species diagnosis.

There are several explanations for shared mtDNA haplotypes between *C. occidentalis*, *C. biennis*, and *C. lambertiana*, the first being gene introgression. According to Petit and Exoffier (2009), loci under high levels of gene flow are less likely to suffer from introgression and species delimitation is best performed using markers associated with the sex that has the most dispersal. Since long-range migration by spruce budworm is heavily female biased (Greenbank *et al.*, 1980), there should be less potential for mtDNA introgression, as mtDNA is maternally inherited. On the other hand, short-range dispersal is more likely to be male influenced, due to males searching for calling females. Therefore, mtDNA introgression could be more frequent among species with sympatric ranges, such as the western *Choristoneura* species, where males disperse short distances to track pheromone plumes.

Hybrid speciation through genomic recombination could also account for sharing of mtDNA haplotypes among the western species, if new species were formed through interspecific hybridization events without individual gene loci having had the opportunity to diverge from one or both parent species (Mallet, 2007; Melo *et al.*, 2009). Hybrid speciation is plausible for the spruce budworm complex, as the ability to hybridize is well established (Harvey, 1997) and evidence of hybrid zones has been found in nature (Volney *et al.*, 1984; Powell, 1995). Although the genomic integrity of recombinant species can be overcome through constant gene flow from the parental species (Coyne and Orr, 2004), there is an increased probability that a hybrid species will persist if it can inhabit an environment where the parental species do not live or have decreased fitness (Buerkle *et al.*, 2000; Mallet, 2007). Species in the spruce budworm complex are strongly associated with different larval host plants and bioregions, with consequent differences in elevation and overall climate. An increase or change in ecological fitness through genetic recombination may have allowed hybrid species to persist in these different niches over time. The present study did not determine the likelihood of this scenario, but does suggest it as a promising avenue for further research.

Sharing of mtDNA haplotypes among species may also be explained by incomplete lineage sorting, or retained ancestral polymorphism, where allele variation precedes a speciation event and has not yet been lost due to random drift or selection. This is especially probable with newer species, as there is a progression over time from polyphyly to paraphyly to monophyly between sister species until eventually most gene trees match the species tree (Funk & Omland, 2003, Omland *et al.*, 2006). Incomplete lineage sorting of mtDNA can be a particularly important factor for rapidly radiating taxa (Funk & Omland, 2003) and has been studied extensively in taxa such as cichlid fishes (e.g. Moran & Kornfield, 1993; Genner & Turner, 2005), Darwin's finches (e.g. Freeland & Boag, 1999; Sato *et al.*, 1999) and sticklebacks (e.g. Rundle *et al.*, 2000). Although the propensity of the spruce budworm complex to undergo rapid radiation is currently unknown, there is potential for rapid ecological or hybrid speciation based on the close ecological and behavioural connection of biotypes to their host plant (Schluter, 2001, 2009; Drès and Mallet, 2002; Coyne and Orr, 2004; Hendry *et al.*, 2007). These mechanisms of speciation have been supported in other insects, including *Rhagoletis* species (Bush, 1969; Feder *et al.*, 1994), pea aphids (Peccoud *et al.*, 2009), soapberry bugs (Carrol & Boyd, 1992), codling moths (Phillips & Barnes, 1975) and walking sticks (Nosil *et al.*, 2008).

A further explanation for shared mtDNA haplotypes among the western species is that I chose a poor marker location within COI. This could be due to stochastic error within a specific region that results in an independent pattern of divergence from that of the entire mtDNA. However, this is an unlikely scenario, as the 470 bp region that I used was within the most informative region of COI based on previous studies (Sperling & Hickey, 1994; Roe & Sperling, 2007). I also compared the tree topology and average sequence divergence for the 2300 bp region of COI and COII, the 658 bp barcode region of COI and the 470 bp region of COI used by Sperling and Hickey (1994) on a limited number of individuals to determine which of the shorter fragments to use. I found that all three tree topologies were identical, yet the average sequence divergence for the 470 bp region was more than twice that of the barcode region. Therefore, the 470 bp

region should provide a typical tree topology, yet also provide more information among and within species due to higher percent sequence divergence.

The *C. fumiferana* complex comprises an appropriate model for evaluating different approaches to cryptic species delimitation because so much research has already been undertaken to understand the life history traits and other biological differences of the species within this complex, thereby establishing the species-level status of these populations. The results from this study indicate that sole reliance on mtDNA could mislead our understanding of the ecological, behavioural and morphological processes of speciation, as different species units are indicated in the *C. fumiferana* complex using mtDNA than are distinguished using a combination of various other traits, including morphology. This is of particular concern for this group, as some species are of more economic importance than others and grouping them together or misidentifying them could lead to poor forest management decisions.

This study also supports the use of character combinations to demonstrate the genomic integrity of taxa when reproductive isolation is either unknown or incomplete and DNA introgression is plausible. Such practice is consistent with both the genomic integrity species concept (Sperling, 2003) and the genotypic cluster species concept (Mallet, 1995), in which population assemblages of individuals with genetic continuity can be used to indicate species units without requiring complete reproductive isolation. On the basis of these species concepts, combining morphology, ecology, behaviour and genetic traits in an integrated taxonomic approach indicates that the five species studied within the *C. fumiferana* complex are different entities. To test further the application of these species concepts, I am undertaking studies to expand the geographical range, the number of species, the number of specimens per species and the variety of characters to evaluate further the taxonomy and relationships of species within the *C. fumiferana* complex.

In conclusion, combining morphometric and mtDNA character sets increases the accuracy of species identifications in comparison with using either character set alone. This supports the use of an integrative method for species

delimitation within cryptic species complexes. Furthermore, when used alone, the colour-based morphometric methods described here provided better discrimination among species than did mtDNA as a sole source of characters, belying the apparent inadequacy of morphology in cryptic species recognition.

Table 2-1. Summary of samples, arranged by species. Number of individuals per haplotype is given in parentheses.

Species Locality	Collection Date	Lat.	Long.	Elev. (m)	Larval Host	Sampling Method	Sample Size (n)	Haplotypes (number of individuals)
C. fumiferana								
CAN: AB: Wolf Lake	2.vii.2006	54.707	-110.968	585	*Picea glauca*	Reared	5	f1 (3), f2 (1), f11 (1)
CAN: AB: Rainbow Lake	15.vi.2005	58.297	-119.404	532	*Picea glauca*	Reared	9	f1 (7), f9 (1), f17 (1)
CAN: AB: East Sousa Creek	15.vi.2005	58.591	-118.495	393	*Picea glauca*	Reared	10	f1 (7), f3 (1), f10 (1), f17 (1)
CAN: AB: La Crete Ferry	16.vi.2005	57.982	-117.113	318	*Picea glauca*	Reared	1	f1 (1)
CAN: AB: Lawrence Lake	17.vi.2005	54.993	-113.661	658	*Picea glauca*	Reared	1	f17 (1)
CAN: AB: Ft McMurray Center	20.vi.2005	56.715	-111.348	243	*Picea glauca*	Reared	2	f1 (1), f2 (1)
CAN: AB: Ft McMurray North	21.vi.2005	56.735	-111.407	281	*Picea glauca*	Reared	5	f1 (4), f17 (1)
CAN: AB: Ft McMurray South	21.vi.2005	56.686	-111.355	385	*Picea glauca*	Reared	4	f1 (3), f17 (1)
CAN: AB: Ft McMurray- 120 km S	21.vi.2005	55.803	-112.186	685	*Picea glauca*	Reared	1	f1 (1)
C. pinus								
CAN: AB: Bellis South	15-25.vii.2006	54.095	-112.128	664	*Pinus banksiana*	*pinus* lure	5	p1 (4), p3 (1)
CAN: AB: Bellis West	15-25.vii.2006	54.120	-112.165	659	*Pinus banksiana*	*pinus* lure	5	p1 (2), p4 (1), p6 (1), p7 (1)
CAN: AB: Smoky Lake Sands W	15-25.vii.2006	54.050	-112.318	617	*Pinus banksiana*	*pinus* lure	5	p1 (3), p5 (1), p8 (1)
CAN: AB: Smoky Lake Sands E	15-25.vii.2006	54.065	-112.285	612	*Pinus banksiana*	*pinus* lure	5	p1 (4), p4 (1)
CAN: AB: Bellis Southeast	15-25.vii.2006	53.940	-111.982	640	*Pinus banksiana*	*pinus* lure	5	p1 (4), p3 (1)
CAN: AB: Bellis West 1	1.vii.2008	54.112	-112.190	638	*Pinus banksiana*	Reared	1	p8 (1)
CAN: AB: Moose Lake	1.vii.2008	54.246	-111.005	560	*Pinus banksiana*	Reared	4	p1 (4)
CAN: AB: Bellis West 2	1.vii.2008	54.117	-112.169	654	*Pinus banksiana*	Reared	2	p1 (1), p7 (1)
C. occidentalis								
CAN: AB: Hillcrest Mine Road	10.vii.2005	49.559	-114.324	1223	*Pseudo. menziesii*	Reared	7	o1 (1), o2 (4), o5 (2)
CAN: BC: Sparwood	10.vii.2005	49.725	-114.850	1148	*Pseudo. menziesii*	Reared	5	o2 (3), b1 (2)
CAN: BC: Ft. Steele	10.vii.2005	49.649	-115.566	828	*Pseudo. menziesii*	Reared	1	o2 (1)
CAN: BC: Invermere	11.vii.2005	50.514	-116.059	969	*Pseudo. menziesii*	Reared	1	b1 (1)
CAN: BC: Wilmer	11.vii.2005	50.557	-116.073	917	*Pseudo. menziesii*	Reared	3	o1 (2), o2 (1)
C. biennis								
CAN: AB: Mount Sarrail	5.vii.2005	50.618	-115.122	1692	*Picea engelmannii*	Reared	5	o1 (2), b1 (3)
CAN: BC: Kootenay / Banff border	11.vii.2005	51.226	-116.054	1640	*Picea engelmannii*	Reared	1	b1 (1)
CAN: AB: West Spray Lakes	12.vii.2005	50.991	-115.372	1678	*Picea engelmannii*	Reared	3	b1 (3)
CAN: AB: Buller Mountain	12.vii.2005	50.868	-115.354	1765	*Picea engelmannii*	Reared	4	o11 (1), b1 (3)
CAN: AB: Sawmill	12.vii.2005	50.749	-115.245	1799	*Picea engelmannii*	Reared	2	o2 (1), b1 (1)
CAN: AB: Wedge Pond	12.vii.2005	50.874	-115.147	1521	*Picea engelmannii*	Reared	1	o2 (1)
C. lambertiana								
CAN: AB: Skyline Road	29.vi.2008	49.970	-114.087	1707	*Pinus contorta*	Reared	2	o1 (1), o2 (1)
CAN: BC: Hosmer	29.vi.2008	49.606	-114.950	1065	*Pinus contorta*	Reared	6	o1 (5), b1 (1)

Table 2-2. Description of morphometric characters measured using ImageJ v1.38x (Rasband, 2006) as shown for a forewing of *C. biennis* in Figure 2-2. Three colour measurements (mean red, blue and green) were derived from each of the first 11 wing areas.

Band/Patch Colouration:
1 Basal patch
2 Median band between longitudinal bar and costal margin
3 Subapical patch along costal margin
4 Subapical patch within middle of wing
Groundcolour:
5 Submedian band from costal edge to middle of wing
6 Costal spot
7 Subterminal spots
Line/Bar colouration:
8 Between submedian and median bands
9 Between costal spot and subapical patch
10 Longitudinal bar
Overall Wing Colouration:
11 Entire forewing
12 Dark scales within entire forewing
Length/Width/Area:
13 Area of entire forewing, excluding fringe
14 Forewing width - from middle of costal spot to anal margin at minimum width
15 Forewing length - from middle of basal margin to terminal margin of wing at maximum length, excluding fringe
16 Length between basal margin and submedian band (straight line) along costal margin
17 Width between costal margin and top edge of submedian band (equals 0 in Figure 2-2)
18 Length of submedian band along costal margin
19 Length of median band along costal margin
20 Length between costal margin and top edge of costal spot (equals 0 in Figure 2-2)
21 Length of costal spot along costal margin
22 Length from terminal end of costal spot to terminal end of wing, below region where wing rounds off and excluding fringe
23 Length between costal spot & postmedian band at minimum separation
24 Length between submedian & postmedian bands along longitudinal bar, at minimum separation
25 Area of costal spot

Table 2-3. MtDNA haplotypes, based on the 470 bp fragment analyzed in this study, with corresponding GenBank accession numbers and references.

Haplotype	Accession No.	References
f1	L19098[b]	Sperling & Hickey, 1994; Roe & Sperling, 2007
f2	GQ890278[ab]	Sperling & Hickey, 1994; Roe & Sperling, 2007
f3	GQ890279[a]	Sperling & Hickey, 1994
f9	GQ890280	Previously unpublished
f10	GQ890281	Previously unpublished
f11	GQ890282	Previously unpublished
f17	GQ890283	Previously unpublished
p1	L19095[b]	Sperling & Hickey, 1994; Roe & Sperling, 2007
p3	GQ890284	Previously unpublished
p4	GQ890285	Previously unpublished
p5	GQ890286	Previously unpublished
p6	GQ890287	Previously unpublished
p7	GQ890288	Previously unpublished
p8	GQ890289	Previously unpublished
o1	L19094, DQ792584[b]	Sperling & Hickey, 1994; Roe & Sperling, 2007
o2	GQ890290[a]	Sperling & Hickey, 1994
o5	GQ890291[a]	Sperling & Hickey, 1994
o11	GQ890292	Previously unpublished
b1	DQ792587[b]	Sperling & Hickey, 1994; Roe & Sperling, 2007
c1	GQ890293[b]	Previously unpublished
m1	GQ890294[b]	Previously unpublished
r2	GQ890295[a]	Sperling & Hickey, 1994

[a] Previously published but not submitted to GenBank.

[b] Sequence analyzed for 2.3 kb COI and COII region, 658 bp barcode region, and 470 bp region previously used by Sperling and Hickey (1994).

Table 2-4. Range of morphometric character values for each species. Colour is measured in RGB, with each of red, green and blue having a possible range of 0 to 255.

Character and Description		Unit	Species				
			C. fumiferana	*C. pinus*	*C. occidentalis*	*C. biennis*	*C. lambertiana*
1R	Red mean of basal patch	RGB	30.68 - 112.14	59.77 - 117.13	64.64 - 136.41	37.44 - 98.65	69.63 - 114.79
1G	Green mean of basal patch	RGB	21.03 - 64.29	17.54 - 56.01	26.66 - 76.04	16.98 - 69.04	23.38 - 61.51
1B	Blue mean of basal patch	RGB	9.86 - 40.49	2.99 - 21.91	9.07 - 36.58	5.43 - 42.74	6.60 - 24.43
2R	Red mean of median band	RGB	32.63 - 116.34	52.01 - 115.31	39.18 - 123.18	30.31 - 99.53	58.83 - 139.92
2G	Green mean of median band	RGB	21.84 - 70.31	17.48 - 61.16	19.96 - 63.00	18.63 - 56.57	20.60 - 75.2
2B	Blue mean of median band	RGB	9.86 - 43.27	3.80 - 25.64	5.65 - 29.21	6.39 - 33.02	5.95 - 32.63
3R	Red mean of subapical patch (costal margin)	RGB	20.70 - 112.32	32.66 - 103.02	44.07 - 104.89	21.20 - 109.15	45.69 - 98.22
3G	Green mean of subapical patch (costal margin)	RGB	15.59 - 75.32	16.59 - 55.48	22.49 - 53.82	15.54 - 74.53	15.07 - 35.2
3B	Blue mean of subapical patch (costal margin)	RGB	6.24 - 45.90	3.52 - 22.16	3.64 - 20.54	5.33 - 41.42	4.04 - 10.76
4R	Red mean of subapical patch (mid wing)	RGB	19.65 - 117.21	31.33 - 111.36	46.41 - 118.51	28.86 - 92.58	50.88 - 109.55
4G	Green mean of subapical patch (mid wing)	RGB	13.96 - 74.48	12.49 - 52.55	18.18 - 56.79	19.57 - 59.72	17.28 - 45.18
4B	Blue mean of subapical patch (mid wing)	RGB	5.20 - 43.19	1.52 - 14.97	5.45 - 26.42	8.23 - 36.75	4.37 - 14.73
5R	Red mean of submedian band	RGB	53.31 - 131.40	79.97 - 151.61	65.77 - 159.72	58.76 - 132.85	71.72 - 152.45
5G	Green mean of submedian band	RGB	23.60 - 101.91	44.43 - 117.23	42.74 - 114.88	44.27 - 105.99	47.62 - 107.75
5B	Blue mean of submedian band	RGB	22.06 - 72.44	16.97 - 77.37	24.37 - 74.73	28.24 - 77.65	20.72 - 64.15
6R	Red mean of costal spot	RGB	38.63 - 147.63	111.89 - 166.55	51.39 - 173.32	84.54 - 149.82	62.69 - 166.97
6G	Green mean of costal spot	RGB	24.62 - 120.09	73.85 - 141.04	35.42 - 140.62	64.41 - 121.42	19.36 - 124.84
6B	Blue mean of costal spot	RGB	11.41 - 89.52	40.92 - 107.24	18.46 - 100.70	41.90 - 87.64	5.52 - 82.03
7R	Red mean of subterminal spots	RGB	31.90 - 138.53	55.39 - 145.94	55.57 - 159.64	61.00 - 111.82	80.52 - 131.71
7G	Green mean of subterminal spots	RGB	24.64 - 97.87	29.95 - 107.03	40.84 - 115.65	37.07 - 88.11	53.19 - 84.46
7B	Blue mean of subterminal spots	RGB	13.69 - 55.92	12.26 - 65.48	24.46 - 75.85	16.83 - 60.45	23.42 - 42.37
8R	Red mean of line between submedian & median bands	RGB	14.06 - 102.93	34.71 - 105.47	22.81 - 107.18	23.27 - 82.36	49.97 - 129.79
8G	Green mean of line between submedian & median bands	RGB	11.66 - 60.06	12.60 - 63.78	15.01 - 45.81	16.80 - 40.55	17.92 - 62.1
8B	Blue mean of line between submedian & median bands	RGB	5.56 - 31.07	3.44 - 31.22	7.40 - 19.69	7.45 - 22.75	4.84 - 26.21
9R	Red mean of line between costal spot & subapical patch	RGB	15.21 - 105.26	24.35 - 98.31	23.00 - 100.34	21.26 - 66.93	49.76 - 106.65
9G	Green mean of line between costal spot & subapical patch	RGB	11.94 - 59.96	12.58 - 44.25	11.55 - 40.68	15.89 - 40.70	13.96 - 42.64
9B	Blue mean of line between costal spot & subapical patch	RGB	5.36 - 34.46	2.53 - 18.97	3.72 - 16.08	6.37 - 22.10	2.88 - 15.32
10R	Red mean of longitudinal bar	RGB	19.71 - 96.60	17.36 - 117.82	27.22 - 99.69	23.76 - 73.70	32.95 - 131.33
10G	Green mean of longitudinal bar	RGB	14.87 - 53.07	10.13 - 51.52	17.84 - 46.30	13.75 - 51.01	14.50 - 101.79
10B	Blue mean of longitudinal bar	RGB	5.76 - 34.57	1.91 - 16.85	8.06 - 22.39	4.89 - 29.29	4.86 - 65.86
11R	Red mean of total forewing area	RGB	45.23 - 112.11	47.86 - 118.90	54.94 - 127.79	51.89 - 101.61	62.75 - 125.96
11G	Green mean of total forewing area	RGB	32.59 - 72.51	25.63 - 86.32	34.55 - 78.60	33.33 - 77.69	32.16 - 70.80
11B	Blue mean of total forewing area	RGB	18.00 - 47.04	11.68 - 56.12	18.85 - 44.95	17.85 - 52.05	16.02 - 35.83
12	Area of dark scales	mm^2	0.58 - 16.61	0.03 - 16.10	0.06 - 10.26	1.20 - 10.00	0.02 - 7.44
13	Total forewing area	mm^2	24.09 - 62.83	22.46 - 42.23	21.21 - 54.39	23.01 - 41.87	26.35 - 30.58
14	Forewing width	mm	3.52 - 5.98	3.34 - 4.55	3.18 - 5.28	3.40 - 4.42	3.58 - 3.91
15	Forewing length	mm	8.27 - 12.85	7.78 - 10.95	8.30 - 12.45	8.22 - 11.09	8.43 - 9.22
16	Length between basal margin & submedian band	mm	1.47 - 2.79	1.62 - 2.42	1.46 - 2.99	1.86 - 2.44	1.67 - 2.10
17	Length between costal margin & top edge of submedian band	mm	0.00 - 0.09	0.00 - 0.14	0.00 - 0.14	0.00 - 0.00	0.00 - 0.00
18	Length of submedian band	mm	0.63 - 2.60	0.35 - 1.66	0.54 - 2.37	0.55 - 2.00	0.44 - 1.68
19	Length of median band	mm	0.90 - 3.05	1.18 - 2.47	0.91 - 2.60	1.10 - 2.61	0.71 - 2.22
20	Length between costal edge & top edge of costal spot	mm	0.00 - 0.28	0.00 - 0.00	0.00 - 0.14	0.00 - 0.13	0.00 - 0.00
21	Length of costal spot	mm	0.24 - 1.42	0.34 - 1.69	0.37 - 2.45	0.34 - 1.56	0.81 - 1.73
22	Length from costal spot to terminal end of wing	mm	3.18 - 6.19	3.10 - 4.15	3.07 - 4.94	3.60 - 4.78	3.21 - 4.07
23	Length from costal spot to postmedian band	mm	0.00 - 1.18	0.00 - 0.25	0.00 - 0.59	0.00 - 0.68	0.02 - 0.57
24	Length between submedian & postmedian bands	mm	0.20 - 3.49	0.43 - 1.75	0.54 - 2.13	0.63 - 1.88	0.17 - 1.22
25	Costal spot area	mm^2	0.00 - 1.94	0.34 - 1.41	0.24 - 1.84	0.13 - 1.67	0.40 - 0.85

Table 2-5. Linear discriminant coefficients for the first three discriminant functions under no selection for 47 morphometric characters.

Character and Desciption		LDF-1	LDF-2	LDF-3
Constant		18.55	46.29	-16.97
1R	Red mean of basal patch	9.35	5.94	22.81
1G	Green mean of basal patch	1.27	-26.73	-41.10
1B	Blue mean of basal patch	-5.10	17.00	18.16
2R	Red mean of median band	6.91	-21.91	-10.56
2G	Green mean of median band	-24.09	46.30	25.71
2B	Blue mean of median band	8.60	-20.52	-12.08
3R	Red mean of subapical patch (costal margin)	1.24	28.89	8.29
3G	Green mean of subapical patch (costal margin)	-10.33	-79.88	-16.15
3B	Blue mean of subapical patch (costal margin)	10.23	43.08	4.81
4R	Red mean of subapical patch (mid wing)	9.88	-17.00	5.37
4G	Green mean of subapical patch (mid wing)	-23.36	40.94	-6.35
4B	Blue mean of subapical patch (mid wing)	9.10	-21.68	1.74
5R	Red mean of submedian band	-10.58	-8.27	-6.22
5G	Green mean of submedian band	9.59	0.69	-7.47
5B	Blue mean of submedian band	2.46	0.66	7.19
6R	Red mean of costal spot	16.18	30.05	-29.92
6G	Green mean of costal spot	-35.39	-54.75	56.84
6B	Blue mean of costal spot	20.24	24.52	-26.73
7R	Red mean of subterminal spots	1.68	-17.01	-42.25
7G	Green mean of subterminal spots	-5.96	48.93	63.60
7B	Blue mean of subterminal spots	0.25	-27.65	-21.36
8R	Red mean of line between submedian and median bands	19.86	-2.47	-15.45
8G	Green mean of line between submedian and median bands	-21.88	9.10	25.11
8B	Blue mean of line between submedian and median bands	5.01	-5.77	-8.69
9R	Red mean of line between costal spot and subapical patch	-4.29	-11.34	9.42
9G	Green mean of line between costal spot and subapical patch	-0.79	23.91	-8.80
9B	Blue mean of line between costal spot and subapical patch	1.45	-11.98	-1.13
10R	Red mean of longitudinal bar	-4.43	15.55	-15.12
10G	Green mean of longitudinal bar	24.01	-30.58	7.60
10B	Blue mean of longitudinal bar	-16.30	14.91	4.12
11R	Red mean of total forewing area	-38.65	34.22	67.19
11G	Green mean of total forewing area	98.33	-70.50	-94.46
11B	Blue mean of total forewing area	-48.39	23.89	36.27
12	Area of dark scales	-0.35	-2.69	-0.73
13	Total forewing area	6.28	8.83	-7.60
14	Forewing width	-11.29	27.92	19.38
15	Forewing length	-0.40	-39.74	15.91
16	Length between basal margin and submedian band	-2.80	-5.92	-3.85
17	Length between costal margin and top edge of submedian band	0.88	-0.30	0.05
18	Length of submedian band	-3.64	-2.81	-0.96
19	Length of median band	1.58	-6.05	-2.12
20	Length between costal edge and top edge of costal spot	0.04	0.32	-0.40
21	Length of costal spot	-1.10	1.03	-0.54
22	Length from costal spot to terminal end of wing	-10.24	11.96	-6.85
23	Length from costal spot to postmedian band	-0.21	0.12	0.58
24	Length between submedian and postmedian bands	-0.93	0.46	-1.05
25	Costal spot area	0.28	-0.28	0.15

Table 2-6. Linear discriminant coefficients for the first three discriminant functions for characters chosen under backward stepwise selection.

Character and Description		var-1	var-2	var-3
Constant		24.43	-25.343	-31.619
1R	Red mean of basal patch	10.411	10.339	6.838
1B	Blue mean of basal patch	-4.023	-4.856	-4.076
3R	Red mean of subapical patch (costal margin)	9.218	10.62	-23.25
3G	Green mean of subapical patch (costal margin)	-30.254	-6.169	39.853
3B	Blue mean of subapical patch (costal margin)	15.373	-3.765	-14.195
6R	Red mean of costal spot	18.912	-38.94	-11.232
6G	Green mean of costal spot	-53.116	75.859	19.885
6B	Blue mean of costal spot	29.942	-34.966	-9.176
7R	Red mean of subterminal spots	-18.075	-15.154	18.974
7G	Green mean of subterminal spots	31.302	9.782	-36.191
7B	Blue mean of subterminal spots	-14.779	5.219	16.777
10R	Red mean of longitudinal bar	6.043	-9.791	4.759
10B	Blue mean of longitudinal bar	-6.361	6.418	-3.495
11G	Green mean of total forewing area	2.839	10.723	11.959
12	Area of dark scales	-0.867	1.714	3.269
14	Forewing width	-7.286	-4.105	-5.859

Table 2-7. Resubstitution and leave-one-out evaluation output for morphology, mtDNA and combined data.

Characters Analyzed	Resubstitution Evaluation						Leave-One-Out Evaluation					
	fum	*pin*	*occ*	*bi*	*lamb*	Total	*fum*	*pin*	*occ*	*bi*	*lamb*	Total
Morphology Only												
No selection	97	100	100	100	100	99	76	97	59	63	75	77
Backward stepwise selection	95	94	94	81	100	93	92	94	76	75	50	85
MtDNA Only												
No selection	100	100	65	75	75	89	95	84	65	75	75	83
Backward stepwise selection	100	100	65	75	75	89	95	84	65	75	75	83
Morphology and mtDNA												
No selection	100	100	100	100	100	100	97	97	71	81	88	90
Backward stepwise selection	100	100	100	100	100	100	97	97	71	81	88	90

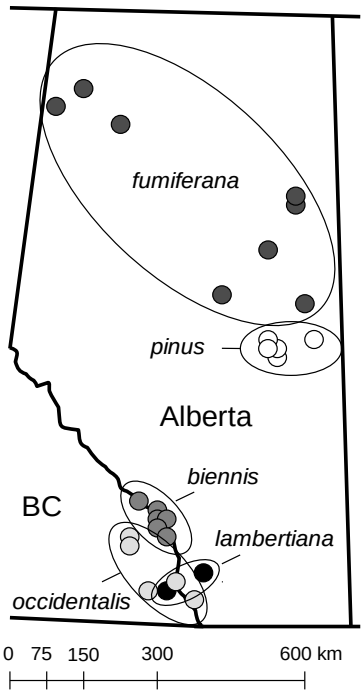

Figure 2-1. Collection locations in Alberta and south-eastern British Columbia for five *Choristoneura* species examined in this study.

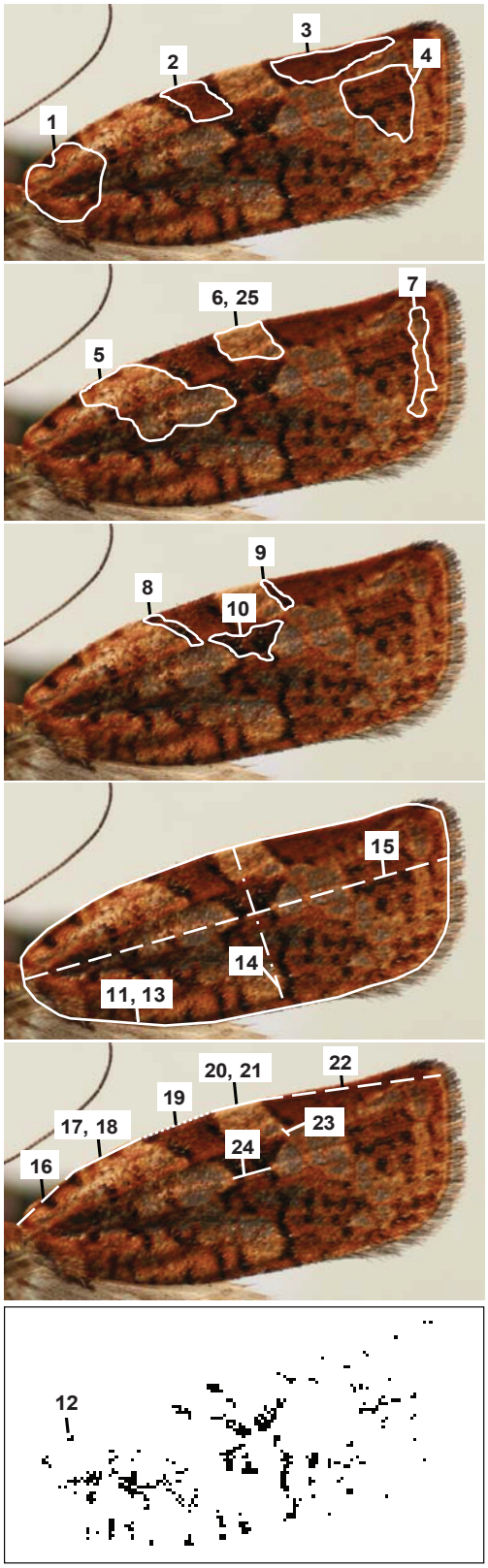

Figure 2-2. Morphometric characters measured using ImageJ v1.38x (Rasband, 2006) as shown for a forewing of *C. biennis*.

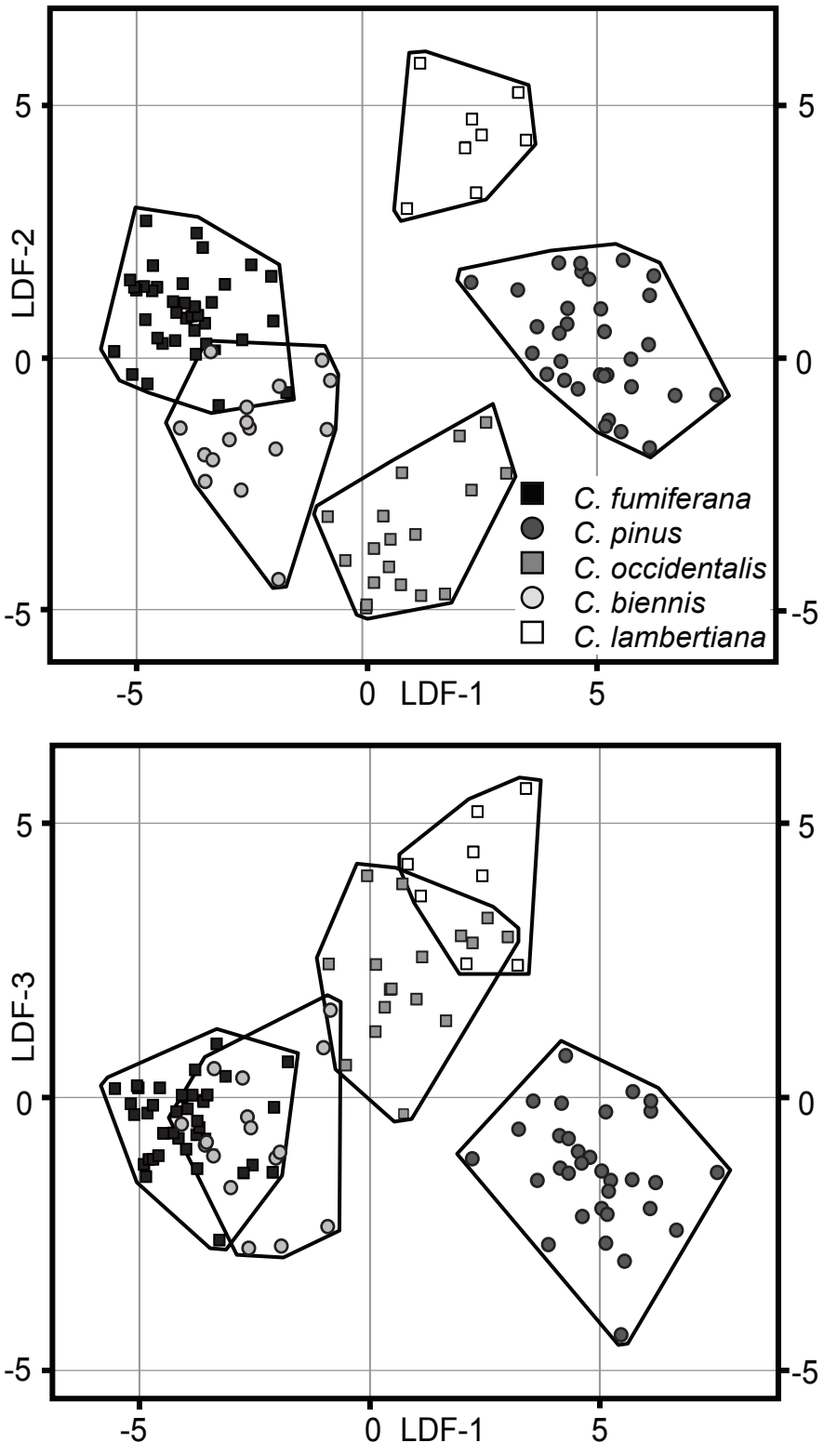

Figure 2-3. Linear discriminant analysis of 47 morphometric characters for five species of the *C. fumiferana* complex.

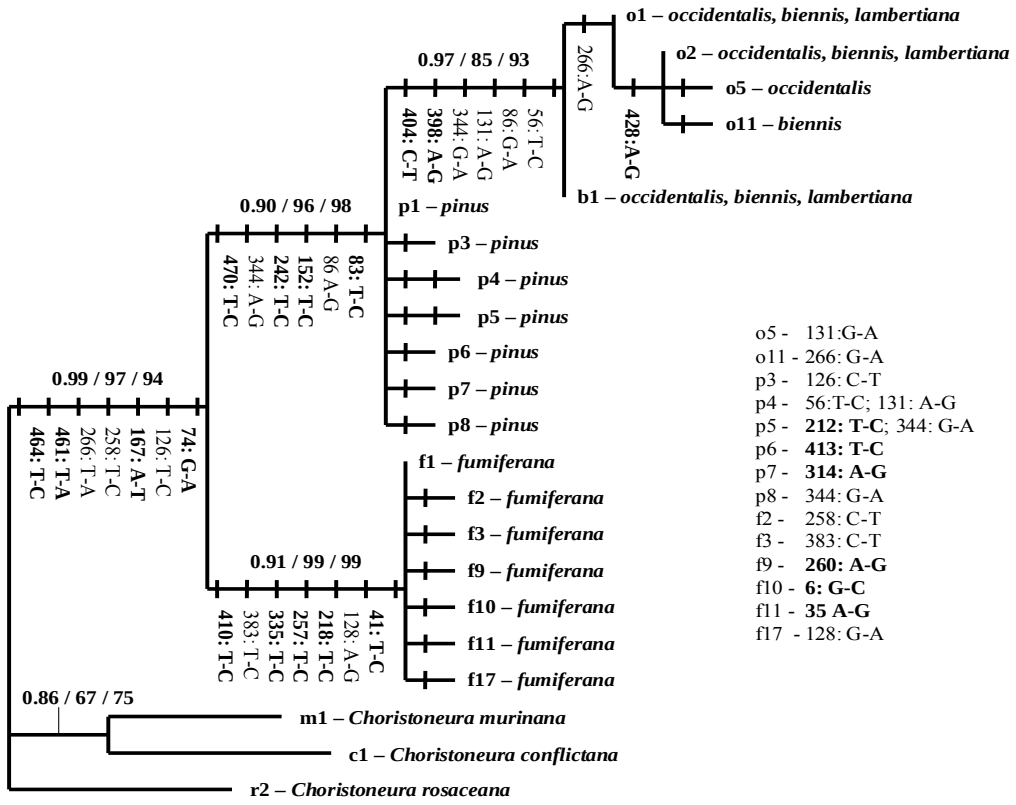

Figure 2-4. Bayesian tree analysed for a 470 bp region of COI mtDNA for all unique haplotypes in the *Choristoneura* study sample. Numbers above the branches are Bayesian support values, maximum parsimony bootstrap values and neighbour-joining bootstrap values, respectively. Crossbars on branches indicate unambiguous character changes, below which is the base pair with ancestral nucleotide listed first. Unique changes are in bold. Autapomorphies for terminal taxa are listed along the right side. Terminal haplotypes list the species that contain them.

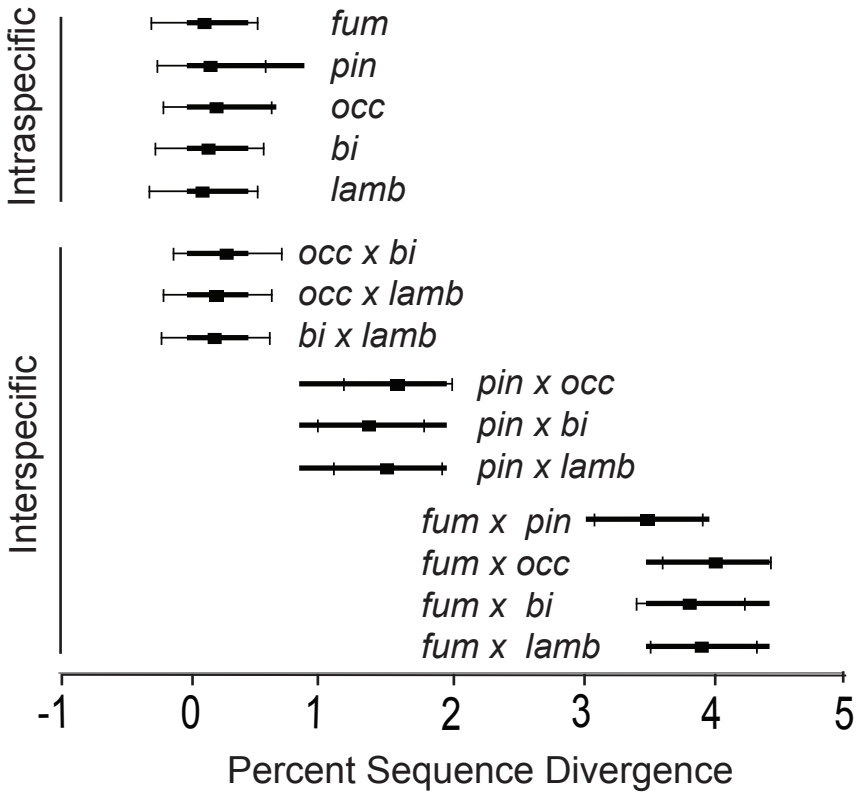

Figure 2-5. Corrected (K2P) intra- and interspecific pairwise differences in mtDNA (470 bp region of COI) among five species of the *C. fumiferana* species complex. Thick black lines, black rectangles, and thin lines with crossbars indicate the range of pairwise differences, mean of pairwise differences, and standard error, respectively.

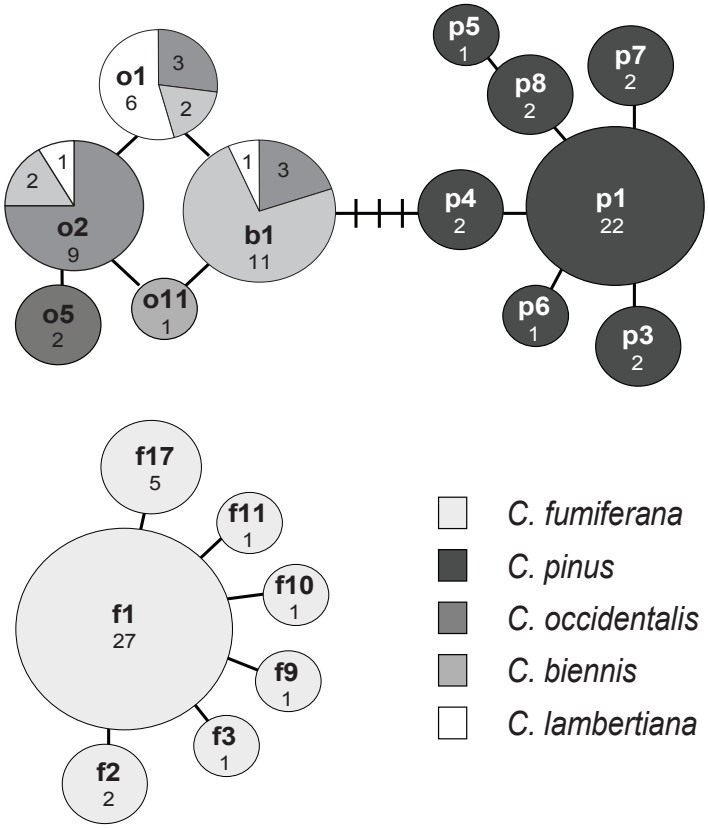

Figure 2-6. Haplotype network generated in TCS v1.21 (Clement *et al.*, 2000) for 19 distinct haplotypes detected within the current study. Circles are labelled with the haplotype name (bold) and number of specimens per haplotype.

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Chapter 3

Isolation and characterization of eight microsatellite loci in the spruce budworm species *Choristoneura fumiferana* and *Choristoneura occidentalis*, and cross-species amplification in related tortricid moths

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The spruce budworm (*Choristoneura fumiferana*) species complex (Lepidoptera: Tortricidae) includes a number of destructive and economically important forest pests native to North America. Larvae feed on foliage, staminate flowers, and cones of coniferous trees leading to decreased growth and ultimately death of the tree if the infestation persists. The taxonomy and evolutionary relationships among species within this group remain unresolved as intra- and interspecific variation in morphological, ecological and behavioural characteristics make it almost impossible to distinguish species using morphology alone. Though known to be difficult to develop for Lepidoptera (Zhang, 2004; Meglécz *et al.*, 2007), I have developed microsatellite markers to study population genetic structure within and between two parapatric species *Choristoneura fumiferana* and *Choristoneura occidentalis*, and other species within the complex.

Genomic DNA libraries were constructed for *C. fumiferana* and *C. occidentalis* using an enrichment protocol (Hamilton *et al.*, 1999). Genomic DNA (gDNA) was isolated from thorax and leg tissue of adults by phenol-chloroform extraction, digested with *Rsa*I, *Alu*I, and *Nhe*I, ligated to SNX linkers, hybridized with biotinylated probes (GT_{12} and $GACA_6$), bound to Streptavidin beads and recovered through amplification with SNX-F. Microsatellite enriched fragments were cloned into pBSIISK$^+$ and transformed into XL1-Blue competent cells.

Inserts from 48 *C. fumiferana* and 47 *C. occidentalis* clones were sequenced on an ABI 3730 DNA Analyser. Sequences were assembled and checked visually for microsatellites in Sequencher 4.0.

Primer pairs were developed for 46 loci using Primer3 (Rozen & Skaletsky, 2000). An M13 tail was added to the 5' end of each forward primer (Schuelke, 2000) and primers were tested on specimens of both *C. fumiferana* and *C. occidentalis*. Eight loci amplified reliably and 5' fluorescently labelled primers were obtained for these loci. I genotyped 32 *C. fumiferana* from Fort McMurray, Alberta, Canada and 24 *C. occidentalis* from Porcupine Hills, Alberta, Canada (Table 3-1) in 15 µL reactions containing 25 ng gDNA, 1x PCR buffer (10mM Tris pH 8.8, 0.1% Triton X-100, 50 mM KCL, 0.16 mg/ml bovine serum albumin), optimized $MgCl_2$ (Table 3-1), 0.2 mM dNTPs, 0.16 µM forward and reverse primers, and 0.1 U/µL *Taq* DNA polymerase. Cycling conditions were: 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 20 s at optimized annealing temperature (Table 3-1), 5 s at 72 °C, followed by 15 min at 72 °C. Fragments were run on an ABI 3730, sized relative to Genescan 500-LIZ and genotyped using GeneMapper 4.0 (ABI).

I calculated summary statistics (Table 3-1) using Excel Microsatellite Toolkit (Park, 2001). Allele numbers ranged from 5 to 29 for *C. fumiferana* and 4 to 20 for *C. occidentalis* with an average of 13.88 and 10.75, respectively (Table 3-1). Observed and expected heterozygosities ranged from 0.35 to 0.74 and 0.67 to 0.95, respectively, for *C. fumiferana* and from 0.35 to 0.71 and 0.45 to 0.91, respectively, for *C. occidentalis*. I performed Hardy-Weinberg and linkage disequilibrium tests using Genepop 3.4 (Raymond & Rousset, 1995). After applying a Bonferroni correction, significant deviation from Hardy-Weinberg equilibrium was observed for 5 loci in *C. fumiferana* and 3 loci in *C. occidentalis* (Table 3-1). Three possible explanations for this deviation are: (1) There was cryptic population structure within the populations that I sampled. Spruce budworm has considerable dispersal abilities (e.g. Dickison *et al.*, 1986), and budworm numbers were higher than in previous years for both sampled populations and may have contained mixed endemic and introduced populations;

(2) Power of the HWE test is limited as the number of specimens that I tested is similar to the number of alleles per locus in those loci that were heterozygote-deficient (50% heterozygote-deficient loci had > 18 alleles and 100% had > 9 alleles); (3) The loci may have null alleles as MicroChecker (Van Oosterhout *et al.*, 2004) showed no evidence for scoring error or large allele dropout. Linkage disequilibrium tests revealed that no loci were significantly linked after applying a Bonferroni correction.

I also genotyped 27 specimens from the remaining species within the *fumiferana* complex (*C. pinus*, *C. biennis*, *C. orae*, *C. lambertiana*, *C. retiniana*, and *C. carnana*), 5 additional specimens from the genus *Choristoneura* (*C. conflictana*, *C. rosaceana*, *C. parallela*, *C. argentifasciata*, *C. murinana*), and 1 additional specimen from a related genus in the family Tortricidae (*Clepsis peritana*) (Table 3-2). Species within the *fumiferana* complex amplified successfully for all loci, although 1 locus in *C. pinus* yielded unscorable banding patterns. Loci were polymorphic and alleles were within expected size ranges (Table 3-2). The proportion of successful amplifications per marker for all species was 100% within the *fumiferana* complex, 57.5% for the remaining species within *Choristoneura*, and 0% for *Clepsis peritana*, indicating that reliable amplification declines as phylogenetic distance increases.

These microsatellite loci are sufficiently variable to help detect population differentiation. I will use these loci to examine relationships between and within species within the *Choristoneura fumiferana* species complex.

Table 3-1. Characteristics, summary statistics, and optimal PCR conditions for microsatellite loci from *Choristoneura fumiferana* and *C. occidentalis* , genotyped from 32 and 24 specimens collected from populations in Alberta, Canada. Primer sequence, repeat motif, optimal annealing temperature (T_a) and $MgCl_2$ concentration for PCR amplification are given for each locus. Number of individuals successfully amplified (n), number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosity, and size range are given for each locus and species. Observed heterozygosities denoted with '*' deviate from Hardy-Weinberg.

					C. fumiferana				***C. occidentalis***			
Locus / Genbank No.	**Primer sequence (5'-3')**	**Repeat motif**	**T_a (^oC)**	**$MgCl_2$ (mM)**	**n**	**N_a**	**H_OH_E**	**Size Range (bp)**	**n**	**N_a**	**H_OH_E**	**Size Range (bp)**
FumT1	PET-GGCTGGAAGAACATATAACC	$(TGA)_6$	50	1.5	31	5	0.74	243-255	22	4	0.55	246-252
FJ542025	GGCTGTTCTGTAAGAATAAACA						0.73				0.63	
Fum18	VIC-TTTTCTTTAAATTCTTCGCTTCA	$(CAGA)_{18}$	56	3.5	32	10	0.69	118-162	24	11	0.42*	102-200
FJ542026	GCCAACGGGAGCCTATTA						0.78				0.84	
Fum37	VIC-GTTCATTGTGAAGTTTGTTTTT	$(AC)_{13}$	49	2.5	32	13	0.63*	89-123	24	8	0.63	92-105
FJ542027	TCGTTAAGGATACTACTAAGAGGA						0.87				0.71	
Occ1	NED-TACGACAGACTTGCTTTCAT	$(TG)_{29}$	56	3.5	23	18	0.35*	253-411	24	19	0.50*	249-461
FJ542028	CACATTTCTTGTGGAAACAG						0.94				0.91	
Occ5	6-FAM-TTCAAGAGATAAAGCCCTGT	$(GACA)_6$	50	1.5	32	7	0.59	165-208	24	9	0.71	192-212
FJ542029	ATCTCACCCTTTCAGCAATA						0.75				0.85	
Occ27	NED-TATTCGTGCAAAATAACAGC	$(GACA)_6$	50	1.5	29	9	0.38*	162-230	23	6	0.35	192-206
FJ542030	CGAAAACCATATTGTTCAAA						0.67				0.45	
Occ29	PET-TACCCCATTTTGGAATACA	$(TG)_{21}$	52	1.5	32	20	0.50*	159-231	24	9	0.46	155-172
FJ542031	GGCTACGGTCTTATTTGTCT						0.94				0.7	
Occ32	VIC-GCTAAGTCCCAGTGGAGATA	$(AC)_{15}$	56	3.5	31	29	0.58*	191-254	24	20	0.58*	196-231
FJ542032	TCGTTCGTGTTTGTATGATT						0.95				0.9	

Table 3-2. Amplification of microsatellite primers originally developed for *Choristoneura fumiferana* and *C. occidentalis* in 12 other related species. Sample size (n) is listed for each species. The size range of products (in basepairs) and number of alleles (in parentheses) are listed for each locus. Successful amplification with an unscorable banding pattern or no amplification are denoted with '+' or '-' respectively.

Species	n	FumT1	Fum18	Fum37	Occ1	Occ5	Occ27	Occ29	Occ32
C. pinus	5	241-255 (3)	201	92-150 (5)	267-404 (2)	200-208 (2)	201-300 (2)	+	211-261 (5)
C. biennis	5	246-249 (2)	118-142 (5)	93-103 (5)	254-290 (8)	200-208 (4)	198-246 (3)	156-165 (5)	201-230 (6)
C. orae	5	246-249 (4)	118-142 (4)	93-105 (4)	252-271 (7)	198-209 (6)	198-202 (2)	156-163 (4)	196-203 (6)
C. lambertiana	3	246-249 (3)	130-170 (3)	93-95 (2)	269-403 (5)	200-204 (2)	197-205 (3)	159-165 (2)	200-215 (2)
C. retiniana	5	252-249 (4)	118-126 (2)	93-97 (3)	249-466 (5)	192-208 (2)	199	169-202 (2)	197-215 (8)
C. carnana	4	246-252 (4)	118-158 (4)	93-99 (3)	253-263 (5)	192-212 (5)	198	156-163 (4)	201-211 (5)
C. conflictana	1	-	-	+	-	-	+	164	-
C. rosaceana	1	-	205	117-134 (2)	+	293	205	+	+
C parallela	1	-	-	87	+	306	+	+	203
C. argentifasciata	1	-	-	-	-	-	+	164-179 (2)	216
C. murinana	1	-	193	152	-	-	205-208 (2)	-	255
Clepsis peritana	1	-	-	-	-	-	-	-	-

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Chapter 4

Relative utility of microsatellites, mtDNA, and adaptive traits for species delimitation in the spruce budworm (*Choristoneura fumiferana*) species complex

Introduction

In population and conservation genetics there is rising awareness that neutral markers, generally considered to include mitochondrial DNA (mtDNA) and simple sequence repeats (SSRs, also referred to as microsatellites), may not accurately represent populations or evolutionary significant units (ESUs), since they often do not reflect variation and divergence in the adaptive traits that are important for defining species (Crandall *et al.*, 2000; Hedrick, 2001; McKay & Latta, 2002; van Tienderen *et al.*, 2002; Holdegregger *et al.*, 2006). Several studies have compared types of markers and concluded that there is very little, if any, correlation between neutral molecular markers and adaptive traits (e.g. Pfrender *et al.*, 2000; Reed & Frankham, 2001; Gomez-Mestre & Tejedo, 2004). This may be particularly problematic for both recently diverged species groups and well defined species that still maintain some gene flow even if they diverged millions of years ago (Price & Bouvier, 2002; Mallet, 2005, 2008).

My objective was to determine the extent of congruence between such putatively neutral markers and adaptive traits in species delimitation within the spruce budworm (*Choristoneura fumiferana*) species complex. This group is a coniferophagous pest complex restricted to the Nearctic region, and contains one member, *Choristoneura fumiferana* Clemens, that is considered North America's most destructive insect defoliator (Volney & Fleming, 2007). *Choristoneura fumiferana* has consequently become a model organism for studying insect outbreak dynamics (e.g.; Greenbank *et al.*, 1980; Royama, 1984; Williams & Liebhold, 2000; Royama *et al.*, 2005; Cooke *et al.*, 2007; Régnière & Nealis,

2007). Ecogeographical, morphological, and behavioural differences have been used to split the *C. fumiferana* species group into thirteen currently recognized species and subspecies (Freeman, 1967; Powell, 1995). Many, if not all, of these differences may be adaptive, and within this study we consider bioregion, host plant association, adult forewing colouration, pheromone attraction, and larval diapause characteristics as adaptive characters.

The adaptive nature of these traits has generally only been demonstrated indirectly or in a general sense. The success of Lepidoptera is often dependent upon the ability of species or populations to survive in their climatic and biotic environment, which is interrelated with their behaviour (Powell & Opler, 2009). Host plant selection is made by adult females using chemical and tactile cues, and this choice is considered to be inherited genetically (Powell & Opler, 2009). The larvae also detect stimuli from the host plant through chemoreceptors on the antennae and mouthparts and will often starve if a specific host plant is not available (Powell & Opler, 2009). As a specific example in *Choristoneura*, Nealis and Lomic (1994) have shown that the rate of establishment and survival of pine-feeding *C. pinus* larvae is dependent on pollen cones and that establishment on vegetative buds is very poor for this species until later stages in larval development. However, spruce and fir feeding species establish as early larval instars on vegetative buds. Adult forewing colouration is also thought to be adaptive as the majority of species are cryptically coloured gray, brown, rust or tan in the family Tortricidae (Powell & Opler, 2009). Pheromone specificity is generally species specific in Lepidoptera and parameters such as chemical blend ratios and time of pheromone release are thought to be maintained by selection pressures (Cardé & Baker, 1984; Silk & Kuenen, 1988). Diapause characteristics may also be adaptive, as the selection and maintenance of second larval diapause in *Choristoneura* has been associated with cool temperatures during insect development (Shepherd, 1961).

Although extensive taxonomic effort has been invested in delimiting and defining the species within this complex, species boundaries remain unresolved. Many taxonomic studies have been undertaken to better identify the species of the

spruce budworm complex. These include studies on their morphology for every stage of development (MacKay, 1953, 1962; Freeman, 1967; Harvey & Stehr, 1967; Harvey, 1983; Volney *et al.*, 1983, 1984; Dang, 1985, 1992; De Benedictis, 1995; Lumley & Sperling, 2010), behavioural traits (Harvey, 1967, 1997; Stehr, 1967; Volney *et al.*, 1984; Silk & Kuenen, 1988; Powell, 1995; Powell & De Benedictis, 1995; Shepherd *et al.*, 1995), ecogeographical characteristics (Stehr, 1967; Shepherd *et al.*, 1995), and genetic characters (Ennis, 1976; Castrovillo, 1982; Sperling & Hickey, 1994, 1995; Harvey, 1996; Lumley & Sperling, 2010). Yet, these species remain difficult to identify because their defining characters differ in frequency rather than being complete character replacements (Harvey, 1985; Dang, 1992; Harvey, 1997).

Powell (1995) describes the taxonomic status of the spruce budworm complex succinctly: 'Probably we know more about the host plant selection, growth, overwintering survival rates, pheromone chemistry, consequences of control measures, sampling methods of all stages, and parasites and predators of this moth than of any other in North America, if not the world. Yet in parts of its range, we do not know its name.' Knowing the names means knowing the species boundaries and being able to identify individuals correctly, whereas not knowing the names can have significant consequences for both pest management and research endeavours. Spruce budworm species differ significantly in their ecological and behavioural characteristics and these traits often imply distinct management considerations. Considering that thousands of projects and people have focused on the spruce budworm complex (McKnight *et al.*, 1988), piecing this information together is tedious enough without the added complexity of species misidentification.

Various technical developments have occurred since the last broad-scale attempts to delimit species in the *Choristoneura fumiferana* complex (Powell, 1995; Harvey, 1996), including the identification of eight SSR loci that cross-amplify among spruce budworm species (Lumley *et al.*, 2009). Although SSR markers have typically been used in intraspecific population studies, I employed them here, along with mtDNA, to determine their utility for delimiting closely

related species sampled across their known geographic ranges. My aim was to determine whether putatively neutral molecular markers are sufficient for species identification, or if it is necessary to continue to associate individuals of the spruce budworm group with adaptive traits for their correct identification.

Materials and Methods

Sampling and Species Concepts

Specimens of the *C. fumiferana* complex were collected from localities across Canada and the U.S.A. (Table 4-1), representing regions inhabited by each of the currently recognized species. I collected the majority of the specimens used in this study from 2005 to 2008. Additional material came from the Canadian provincial and federal government pheromone trap monitoring programs (2005, 2007), collections by F. Sperling & J. Powell in the western USA, collections made by the Canadian Forest Insect & Disease Survey (FIDS) for projects reported in Sperling & Hickey (1994, 1995), the Mississippi Entomological Museum, and several individual collectors. When possible, samples were collected as larvae from their host plant and reared to adults on host plant material. Samples were also collected using pheromone lures for *C. fumiferana*, *C. pinus pinus* Freeman, and *C. orae* Freeman. Lures for *C. fumiferana* consisted of 95:5 (*E*,*Z*)-11-tetradecenal (Contech, Victoria, BC). For *C. p. pinus*, lures were a 9:1 ratio of 85:15 (*E*,*Z*)-11-tetradecenyl acetate and 85:15 (*E*,*Z*)-11-tetradecen-1-ol (Silk *et al.*, 1985) from the Canadian Forest Service. For *C. orae*, the lures consisted of 82:9:9 (*E*,*Z*)-11 tetradecenyl acetate and *E*-11-tetradecen-1-ol (Gray *et al.*, 1984) from the United States Forest Service. Samples were also collected using blacklight and mercury vapour light traps. Specimens were stored dry or in 100% ethanol and placed in a -20 ^{o}C or -70 ^{o}C freezer. Longitude, latitude, elevation, associated coniferous tree species, and collection date were recorded for each site. Additional information recorded for larval collections included larval host plant, diapause characteristics, and adult emergence date for each individual. Voucher specimens and images have been deposited at the University of Alberta

in the E. H. Strickland Entomological Museum, except for vouchers that were deposited in the Canadian National Collection by Sperling & Hickey (1994, 1995).

Differences in host plant association, pheromones, geographical distribution, larval diapause, and forewing colour and pattern were the original basis for delimitation and description of species, and are still the primary means of identification (Freeman, 1967; Harvey, 1985; Powell, 1995; Shepherd *et al.*, 1995; Lumley & Sperling, 2010). Therefore, these features were used to identify individuals to putative species (Table 4-2).

In examining spruce budworm populations in the western US, Powell (1995) defined species concepts on a 'three-tiered assessment', which included reproductive isolation (pheromone chemistry and related behaviour), ecological separation (larval host plants), and morphology. Authors like Freeman (1958, 1967) and Obraztsov (1962) did not explicitly state which species concept(s) they used in describing the species and subspecies within the complex, but they regularly considered many of the same characters (morphology, geographical distribution, larval host plants, life-history, and other behavioural characters) as did Powell (1995). I prefer concepts that distinguish between the primary species definition and the secondary operational methods used for discovering and delimiting species (e.g. Simpson, 1951, 1961; Wiley, 1978; Frost & Kluge, 1994; Mayden, 1999; de Queiroz, 2007). This allows systematists to focus their energies on determining appropriate characters for delimitation rather than continuing to focus on defining species conceptually (Mayden, 1999; Sites & Marshall, 2003, 2004; Sperling, 2003; Hey, 2006a; de Queiroz, 2007). Based on my current understanding of their ecogeographical, behavioural and morphological traits, spruce budworm species can be delimited in the same manner using these definitions (Freeman, 1967; Powell, 1995). Therefore, I focus here on determining methods for identification of currently recognized species rather than on defining new species boundaries.

Mitochondrial DNA

The full 2.3 kb region of COI and COII for 12 previously sequenced specimens (Roe & Sperling, 2007; Lumley & Sperling, 2010) was included in the phylogenetic analysis. These specimens included at least one individual representing each of the five main mtDNA lineages, as described in Sperling and Hickey (1994), and three outgroup species, including *Choristoneura rosaceana* Harris, *C. conflictana* Walker, and *C. murinana* Hübner. The full 2.3 kb region was included to increase the potential for a stable and accurate basal phylogeny. For the remaining 1155 individuals, which included one additional outgroup specimen (*C. rosaceana*), the 470 bp region of COI mtDNA first described for use in *Choristoneura* by Sperling and Hickey (1994) was amplified and sequenced as in Lumley and Sperling (2010). This region was chosen based on a comparison of the 470 bp region with the 658 bp barcode region (Hebert *et al.*, 2003) and the full 2.3 kb region of the COI and COII genes. The 470 bp region was found to have 2.55% average percent sequence divergence, compared to 1.11% for the 658 bp barcode region and 1.62% for the overall 2300 bp region of COI and COII (Lumley and Sperling, 2010). Therefore, the 470 bp region was deemed to be more informative and cost effective for *Choristoneura* than the adjacent barcode region. In total, 1163 ingroup specimens were sequenced successfully and included in the analysis. Sequence was assembled and checked in Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI), aligned in PAUP 4.0b10 (Swofford, 2003), then reduced to unique haplotypes using MacClade v4.08 (Maddison & Maddison, 2005). New haplotypes were deposited in GenBank and all accession numbers are listed in Table 4-3.

Recent evidence indicates that mtDNA may not be neutral if the majority of base pair differences are non-synonymous rather than synonymous (Galtier *et al.*, 2009; Wares, 2010). I compared all ingroup haplotypes over the 470 bp sequence to determine the percentage of synonymous mutations. As 88% of mutations were synonymous, I considered the 470 bp mtDNA sequence to be primarily neutral for the spruce budworm species complex.

CIPRES portals v1.15 and v2.1 (Miller *et al.*, 2009) were used to analyze the mtDNA sequence using maximum parsimony, maximum likelihood, and Bayesian analysis. Maximum parsimony searches were performed using PAUP 4.0b10 (Swofford, 2003) with a CIPRES wrapper around the PAUP heuristic search command, tree bisection and reconnection branch swapping, and 200 ratchet iterations. Strict and 50% majority rule trees were calculated to generate a final tree. Maximum likelihood searches were done using RAxML v7.0.4 (Stamatakis, 2006) with 1000 rapid bootstrap inferences (Stamatakis *et al.*, 2008), and using the RAxML GTR+G+I model in CIPRES portal v1.15. Bayesian analysis was completed using MrBayes v 3.2.1 (Ronquist & Huelsenbeck, 2003) using the GTR+G+I model, with the Markov-chain Monte Carlo (MCMC) calculation running for 10,000,000 generations, trees sampled every 1,000 generations, and the first 25% of trees being discarded as burnin. Trees were summarized and viewed in Treeview v1.6.6 (Page, 1996). The geographic distributions of the main mtDNA lineages were mapped using DIVA-GIS v5.2 (Hijmans *et al.*, 2005).

Microsatellite Markers

Eight SSR loci developed by Lumley *et al.* (2009) were amplified for the same individuals that were sequenced for the 470 bp region of COI mtDNA, plus some additional specimens that were not sequenced but had previously had DNA extracted for restriction enzyme analysis (Sperling & Hickey, 1995). Amplified product was run on an ABI Prism 3730 Analyzer, sized relative to Genescan LIZ-500, then genotyped using Genemapper 4.0 (ABI). A total of 1135 individuals amplified successfully.

SSR data from the 1135 specimens were analyzed in Structure v2.3.2 (Pritchard *et al.*, 2000) using the admixture model and with four geographic regions being used as sampling location priors. These regions were: 1) southern British Columbia, south-western Alberta and the western US; 2) Rocky Mountains (north of Porcupine Hills, AB), northern British Columbia, Yukon, and Alaska; 3) coastal regions of British Columbia and Alaska; and 4) east of the

Rocky Mountains from Alberta to Newfoundland and the eastern US. One iteration for each population size (k) equalling 1 through 26 was analyzed with MCMC running for 100,000 generations and initial burnin of 10,000 generations to determine when population structure broke down. Based on this analysis, a more thorough analysis was then completed with 10 iterations each of k = 1-10, and with MCMC running for 500,000 generations and initial burnin of 50,000 generations for each iteration. To determine the most likely number of populations, the Structure likelihood results for each k were used to calculate ΔK (Evanno *et al.*, 2005). This calculation uses a combination of the likelihood value plus the stability of the likelihood values for each k to determine the probability of k and, therefore, the most likely number of populations. The geographical distributions of the SSR assignments at k=6 were mapped using DIVA-GIS v5.2 (Hijmans *et al.*, 2005).

The four geographic subsamples (coastal, south-western, northern, and eastern regions) were then analyzed using the same parameters in Structure as for the total sample. This exercise was intended to determine if individuals were assigned more correctly to putative species when analyzed for a smaller geographic range. For these analyses, the geographic regions were further broken down into more specific localities for Structure to use as prior information when assigning individuals to populations.

Combined Data

Following separate analyses on SSRs and mtDNA, data were then combined to determine the congruence between putative adaptive traits and putative neutral alleles. Tentative species identification, based on phenotype information (larval host plant, larval diapause, pheromone attraction, and forewing colour and pattern), was compared qualitatively to mtDNA haplotype and SSR assignment for each individual. Species ranges as per previously published maps (Harvey, 1985) were amended to include current findings and mapped using DIVA-GIS v5.2 (Hijmans *et al.*, 2005).

I was interested in finding intermediate combinations as they may indicate hybridization or mtDNA introgression events between species. For putative species in which there was congruence between adaptive traits and neutral markers (*C. fumiferana*, *C. pinus*, *C. lambertiana* Busck, *C. retiniana* Walsingham), it was possible to detect intermediate combinations in two different ways. First, the number of individuals containing each mtDNA and SSR assignment was recorded for the putative species. Second, each specimen was grouped into one of thirty possible genetic permutations on the basis of a combination of mtDNA lineage and SSR assignment. Through these groupings, specimens with incongruence between their putative species identification, based on adaptive traits, and genetic combination were identified.

Results

Putative Species Identification

Individuals were separated into putative species on the basis of host plant association, pheromone attraction, bioregion, length of larval diapause, and forewing colour and pattern as described in Table 4-2. Two groups, *Choristoneura orae 'Inland'* and *Choristoneura lambertiana 'North'*, were tentatively named to species but left as separate identities because the collected sample information was insufficient for positive identification to the respective described species.

Typically, *Choristoneura orae* is associated with Sitka spruce along the coasts of British Columbia and Alaska. However, *'Choristoneura orae 'Inland'* was collected on lutz or white spruce just inland from typical coastal areas, and was genetically more similar to *C. orae* than to *C. fumiferana*, which were collected in the same or nearby localities. *Choristoneura orae* have previously been collected within the inland region (Sperling & Hickey, 1994; Shepherd *et al.*, 1995) where they were mainly identified by pheromone attraction, a trait unavailable for the *Choristoneura orae 'Inland'* samples that were collected as larvae.

Choristoneura lambertiana 'North' ranged from western Saskatchewan to north-central Oregon. Although found sporadically in jack pine stands, they were mainly associated with lodgepole pine. They were mainly collected using *C. pinus* pheromone lures, but there were also a few samples resembling this group that were collected as larvae from lodgepole pine in western Alberta and south-western British Columbia. The forewing colouration and pattern of some of the specimens in south-western Alberta resembled *Choristoneura lambertiana* nr. *subretiniana* located in the Rocky Mountains, as described by Powell (1995). The remaining specimens in the group were more similar to those previously collected by Gray and others in British Columbia, and may be similar to or the same as those described by Gray & Slessor (1989). Their forewing colouration and pattern is more similar to *C. occidentalis* than to either typical *C. pinus* or *C. lambertiana* (Table 4-2). Overall, based on a combination of morphology, pheromone attraction, host association, and genetics they are most likely a northern form of *C. lambertiana*, and have been tentatively designated as such.

Mitochondrial DNA

In total, 142 unique ingroup COI haplotypes were found, of which 113 were new and 29 were previously published (Sperling & Hickey, 1994; Roe & Sperling, 2007; Lumley & Sperling, 2010). New haplotypes were deposited in GenBank (Table 4-3). Using the parsimony settings in PAUP, excluding the outgroup, and including the full 2.3 kb region of COI and COII analyzed for 9 ingroup haplotypes, 170 characters were parsimony informative, 115 variable characters were parsimony uninformative, and 2015 characters remained constant. When analyzed for the 470 bp region sequenced for all ingroup specimens, 66 characters were parsimony informative, 30 variable characters were parsimony uninformative, and 374 characters remained constant.

Maximum parsimony, maximum likelihood, and Bayesian analysis produced very similar trees and, therefore, only the maximum likelihood tree is shown (Figure 4-1). The analyses yielded five main lineages, being the f-, p-, o-, oβ, and bβ-lineages (Figure 4-1), that corresponded to the five lineages previously

described by Sperling and Hickey (1994). The mtDNA for *C. fumiferana* and *C. p. pinus* formed distinct clades. The western species (*C. occidentalis* Freeman, *C. biennis* Freeman, *C. orae*, *C. carnana* Barnes & Busck, *C. retiniana*, and *C. lambertiana*) and one eastern subspecies (*C. pinus maritima* Freeman) shared haplotypes within the remaining three lineages (o, oβ, and bβ). Figure 4-2 shows the geographic distribution of these five mtDNA lineages.

Microsatellite Analysis

Structure (Pritchard *et al.*, 2000) likelihood results were used to calculate Δ*K* (Evanno *et al.*, 2005), which indicated that the most likely number of populations is two. One of these two populations contained all individuals with the *C. fumiferana* and *C. pinus* phenotypes, and the other contained individuals having phenotypes associated with the remaining six species. However, Structure was allowed to run for up to k=10 and the most species, as defined and identified by their phenotype (Table 4-2), were delimited at k=6 (Figure 4-1, 4-2). Four of these six populations each corresponded with a described species (*C. fumiferana*, *C. pinus*, *C. retiniana*, and *C. lambertiana*). The remaining two SSR populations mainly contained *C. occidentalis*, *C. biennis*, *C. orae*, *C. orae 'Inland', C. carnana*, and *C. lambertiana 'North'*. The main species within these two SSR populations are largely parapatric with little geographical overlap, with the exception of *C. occidentalis* and *C. lambertiana 'North'*, which are attracted to different pheromone blends. Figure 4-2 shows the geographic distribution of the SSR assignments at k=6.

Four geographic subsamples (coastal, south-western, northern, and eastern regions), were also analyzed in Structure to determine whether further delimitation could be accomplished by reducing samples to geographically constrained regions. Additional subpopulations were found in some cases, but no further association was found that indicated congruence between neutral markers and phenotypic traits.

Combined Data

Pheromone attraction, larval host plant, and SSR assignment were mapped beside each mtDNA haplotype (Figure 4-1) to allow a visual assessment of patterns of association. MtDNA and SSR assignment were mapped by locality (Figure 4-2) to allow visual assessment of geographical patterns. Individuals were identified to putative species and associated with each mtDNA and SSR grouping (Table 4-4). The numbers of individuals associated with each mtDNA and SSR combination are given in Table 4-5. Overall, this allowed us to assess associations between the two types of neutral markers as well as between neutral markers and species as assigned by their adaptive traits. Mismatches in classifications also allowed for detection of possible hybridization, mtDNA introgression, or retained ancestral polymorphism.

SSR population 1 and the mtDNA f-lineage were highly associated (Table 4-5). This grouping corresponded exclusively with larvae that fed on white spruce or balsam fir (Figure 4-1), with individuals collected in the boreal region (Figure 4-2, 4-3), with adults attracted to the *C. fumiferana* lure (Figure 4-1), and with adults having the forewing colour and pattern described for *C. fumiferana* (Table 4-2). Overall, these phenotypic traits fit the description for *C. fumiferana* (Table 4-2). Exceptions with f-lineage mtDNA were 2 specimens that were assigned to Population 3 (Western B) and 2 specimens that were assigned to Population 4 (*C. lambertiana*) (Table 4-5). All of these specimens were identified as *C. fumiferana* based on adaptive traits (Table 4-4). Eight specimens were assigned to SSR population 1 (*C. fumiferana*) but contained o-lineage mtDNA (Table 4-5), and were tentatively identified as *C. fumiferana* (n=4), *C. biennis* (n=2), *C. lambertiana 'North'* (n=1), and *C. orae 'Inland'* (n=1) (Table 4-4). Three specimens were assigned to SSR population 1 (*C. fumiferana*) but contained p-lineage mtDNA (Table 4-5), of which all were identified as *C. pinus* (Table 4-4). The majority of these exceptions were collected in Alberta, identifying this region as a likely hybrid zone.

SSR populations 2 and 3 were highly associated with the mtDNA o-lineage. They were also associated with the oβ- and bβ-lineages, though to a lesser

extent due to fewer specimens containing these mtDNA lineages. Individuals that were assigned to these populations were collected throughout western North America (Figure 4-2, 4-3). The phenotypic diversity of individuals within this grouping was high, with larvae collected from white spruce, Engelmann spruce, Sitka spruce, Douglas-fir, subalpine fir, lodgepole pine, and juniper (Figure 4-1), and with adults collected using *C. fumiferana*, *C. pinus* or *C. orae* pheromone traps (Figure 4-1). These phenotypic traits, along with observed adult forewing colour and pattern, were associated with all species found within western North America (*C. occidentalis*, *C. biennis*, *C. orae*, *C. orae 'Inland'*, *C. lambertiana*, *C. lambertiana 'North'*, *C. retiniana*, and *C. carnana*) (Table 4-4). Of these species *C. lambertiana* and *C. retiniana* were the least associated with this grouping due to higher association with SSR populations 4 and 6, respectively, as described below.

SSR population 4 was mainly associated with specimens containing the o-lineage and, to a smaller extent, specimens containing the oβ- or bβ-lineages (Table 4-5). The majority of specimens were assigned to SSR population 4 were identified as *C. lambertiana* (Table 4-4) based on phenotype (Table 4-2). Exceptions include 2 specimens containing the f-lineage that were identified by phenotype as *C. fumiferana*. Based on phenotype, there were also 3 specimens identified as *C. occidentalis* and 6 specimens identified as *C. lambertiana 'North'* that were assigned to SSR population 4 (Table 4-4). Of the 15 specimens identified as *C. lambertiana* that were amplified for SSRss (Table 4-4), the majority were assigned to population 4 (n=14) and the remaining individual was assigned to population 2 (n=1).

SSR population 5 and the mtDNA p-lineage were highly associated (Table 4-5). This grouping corresponded exclusively with larvae that fed on jack pine or eastern white pine (Figure 4-1), with individuals collected in the boreal region (Figure 4-2, 4-3), with adults attracted to the *C. pinus* lure (Figure 4-1), and with adults having the forewing colour and pattern described for *C. pinus pinus* (Table 4-2). Exceptions were 3 specimens that contained the p-lineage but were assigned to population 1 (*C. fumiferana*), and 3 specimens that were assigned to population

5 (*C. pinus*) but contained western mtDNA lineages (o or bβ) (Table 4-5). All of these exceptions were identified as *C. pinus pinus* by their adaptive traits except for one specimen that identified as the subspecies *C. pinus maritima*. Again, these exceptions were mostly collected in Alberta. *C. pinus maritima* was collected in Tennessee.

SSR population 6 was associated with specimens that contained the o, oβ, and bβ lineages (Table 4-5), and it corresponded with individuals identified as *C. retiniana* (Table 4-4) based on phenotype (Table 4-2). The exception was 1 specimen phenotypically identified as *C. occidentalis*. There were also several phenotypic *C. retiniana* individuals that were assigned to SSR population 2 (n=1) or 3 (n=5). All of these exceptions were collected in northern California in a previously identified hybrid zone (Volney *et al.*, 1984; Powell, 1995).

Choristoneura lambertiana 'North' contained o-lineage (n=75) or bβ-lineage (n=13) mtDNA. They were mainly assigned to SSR population 3 (n=66), but some specimens were also assigned to populations 1 (n=1), 2 (n=15) and 4 (n=6). Of interest, the only two localities where specimens were assigned to SSR population 4 were in south-western Alberta and south-eastern British Columbia. The south-western Alberta specimens were collected with *C. pinus* lures over a single night. Within the same trap samples were specimens assigned to SSR populations 2 and 3 that had similar phenotypes to those that were assigned to SSR population 4. Some of these specimens were similar in features to *C. lambertiana* nr. *subretiniana* from the Rocky Mountains as described by Powell (1995). The south-western British Columbia specimens were collected on lodgepole pine, and within the same collection there were larvae that were assigned to SSR population 3. Further work in these areas would be useful to understand the interactions between *C. lambertiana* and *C. lambertiana 'North'*.

Choristoneura orae 'Inland' contained o-lineage (n=33) or bβ-lineage (n=13) mtDNA, and mainly were assigned to SSR population 2 (n=25) or 3 (n=21). One specimen was assigned to population 1 (n=1). Further work in Alaska, the Yukon, and northern British Columbia using both pheromone lures

and larval collections is needed to help resolve the interaction between *C. orae* and *C. orae 'Inland'*.

There were many additional observations that need further work to gain a better understanding of their significance. One interesting observation was that the oβ and bβ mtDNA lineages may be associated with individuals attracted to the *C. fumiferana* and *C. pinus* lures, respectively. More samples with pheromone attraction information that contain these lineages are needed to confirm these findings. Also, in several locations in Montana, I found *C. occidentalis* larvae on juniper which is an unrecorded host plant for coniferophagous *Choristoneura* based on Harvey (1985) and Powell (1995). These specimens were preserved as larvae in ethanol, so it is unknown whether they would have successfully been reared to the adult stage while feeding on juniper as larvae. Larvae were in relatively high numbers on juniper, but it is possible that these records were coincidental. The larvae may have been feeding on nearby (but not overhanging) Douglas-fir, and fallen to the ground, then crawled to the juniper, or they may have been blown by the wind on their silken threads (ballooning).

In several locations I collected larvae from the same host, or adults from the same pheromone trap, that contained different 'pure' genetic types. Of particular note were *C. fumiferana* genetic types (f-lineage mtDNA + population 1 SSR assignment) or *C. pinus* genetic types (p-lineage mtDNA + population 5 SSR assignment) mixed with western genetic types (o-, oβ-, bβ-lineage mtDNA + population 2 or 3 SSR assignment) in inland Alaska, the Yukon, central to western Alberta, western Saskatchewan, and Cypress Hills. Sperling & Hickey (1994) also found two mtDNA lineages at single locations in Fairbanks, AK, Red Lodge Provincial Park, AB, and Cypress Hills, AB. Finer-scaled experiments in these regions of sympatry are needed to further understand the dynamics of gene flow, or the lack thereof, between species.

Discussion

Over the past few decades, systematists have increasingly used molecular markers to help delimit and identify species (Caterino *et al.*, 2000; Sperling & Roe, 2009), with mtDNA being the most popular marker (Galtier *et al.*, 2009). Most molecular markers presently used in systematics and population genetics, such as mtDNA, SSRs, RAPDs (randomly amplified polymorphic DNA), ISSRs (inter simple sequence repeats), AFLPs (amplified fragment length polymorphisms), and allozymes are generally assumed to be neutral (Skibinski *et al.*, 1993; Schlötterer & Wiehe, 1999; Bekessy *et al.*, 2003; Holderegger *et al.*, 2006; Galtier *et al.*, 2009; Sperling & Roe, 2009; Sullivan *et al.*, 2009), although recent evidence suggests that these assumptions may not always hold true (e.g. Watts *et al.*, 2008; Galtier *et al.*, 2009; Wares, 2010). A variety of lines of evidence also indicate that genetic diversity associated with putatively neutral molecular markers may not correspond well with that found for traits associated with life history, behaviour, morphology, or physiology (e.g. Pfrender *et al.*, 2000; Reed & Frankham, 2001; Bekessy *et al.*, 2003; Gomez-Mestre & Tejedo, 2004).

I compared a 470 bp region of mtDNA and eight SSR markers to several adaptive traits that are currently used to identify species within the spruce budworm species complex. This was intended to help us develop a molecular tool using putatively neutral markers for species identification in this complex rather than requiring a combination of morphological, ecological, and behavioural traits. My results indicate that there are varying degrees of congruence between neutral markers and adaptive traits within putative species of the spruce budworm species complex. Congruence of mtDNA and SSR markers with the *C. fumiferana* and *C. pinus* phenotypes is high, making it possible to delimit and identify these species using either of these genetic marker types. Congruence of SSR markers with individuals having the *C. retiniana* and *C. lambertiana* phenotypes is moderately good and can be used to assist in species identification, but mtDNA association is poor, thereby making it difficult to determine the extent of introgression and gene

flow with other species. Congruence with mtDNA and SSR markers is low for the remaining species. Individuals containing the phenotypes described for *C. occidentalis*, *C. biennis*, *C. orae*, *C. orae 'Inland'*, *C. carnana* and *C. lambertiana 'North'* (Table 4-2) share assignments to two SSR populations (k=6). They also share three mtDNA lineages along with *C. retiniana* and *C. lambertiana*, making it impossible to identify or delimit these putative species using the molecular markers tested in this study.

All parapatric and sympatric species show some evidence for natural hybridization and introgression, from very low numbers of individuals in *C. fumiferana* and *C. pinus* to what may be widespread hybridization amongst *C. occidentalis*, *C. biennis*, *C. orae*, and *C. carnana* (Table 4-4, 4-5). Although *Choristoneura* hybrids can be readily produced in the laboratory (e.g. Harvey, 1997), it has been more difficult to determine the extent of natural hybridization in the field. Previous work with RAPDs found evidence for hybridization between sympatric populations of *C. fumiferana* and *C. pinus* in Atlantic Canada (Deverno *et al.*, 1998). Hybrid zones have also been identified in the western United States. Using a combination of morphology, pheromone specificity, life-history, and host association, evidence has been found for a hybrid zone in southern Oregon between *C. occidentalis* and *C. retiniana* (Volney *et al.*, 1983, 1984; Liebhold & Volney, 1984; Liebhold *et al.*, 1984; Volney & Liebhold, 1985). This same region, as well as northern California, Nevada, and Utah, was also found to contain intermediates by Powell (1995), and again I have found evidence for the existence of this hybrid zone using a combination of neutral markers and adaptive traits. Using this combination, my results also indicate that Alberta is a likely hybrid zone for several species that are not typically sympatric but overlap in this region. These overlapping species include *C. fumiferana*, *C. biennis*, *C. occidentalis*, *C. pinus*, and *C. lambertiana 'North'*.

Previous studies indicate that *C. occidentalis*, *C. biennis*, *C. orae*, and *C. carnana* are different species based on ecology, behaviour and morphology (e.g. Freeman, 1967; Harvey, 1967; Powell, 1995; Lumley & Sperling, 2010). However, no successful method has yet been found for their consistent

delimitation across their species ranges using neutral markers (Castrovillo, 1982; Harvey, 1996; Lumley & Sperling, 2010). These four species are largely parapatric, and it may be possible that they represent geographical segregates of a single species and should be given subspecies status. However, there is a taxonomic dilemma in using the traditional binomial or trinomial system of nomenclature; *C. carnana* is currently made up of *C. carnana carnana* Barnes & Busck and *C. carnana californica* Powell, and information regarding their relationship to each other would be lost if they were grouped with *C. occidentalis*, *C. biennis*, and *C. orae* as subspecies. There is also evidence for reproductive isolating mechanisms between species, with *C. orae* having different pheromone specificity compared to the other three species (Harvey, 1985) and *C. biennis* fixed for two-year larval diapause (Nealis, 2005). In consideration of these issues, as well as in the interest of maintaining taxonomic stability, I have left the application of formal taxonomic names for the spruce budworm group as currently practiced by entomologists and foresters. Additional work is needed to determine if there are adaptive markers that associate with the adaptive traits found in these species. If not, then it may be appropriate to change the taxonomic state of these species at that time.

Based on my findings using SSRs, there may be restricted gene flow between *C. lambertiana* and the remaining western species, as well as between *C. retiniana* and the remaining western species. However, this restriction may be relatively recent since they do not form separate lineages using mtDNA. Both of these species are part of the acetate pheromone group (Harvey, 1985), which may allow them to maintain a separation from *C. carnana* and *C. occidentalis*, which are attracted to aldehyde pheromone (Harvey, 1985). *Choristoneura lambertiana* is the only pine feeder in the western regions, which may also help to restrict gene flow with other species. As previously discussed, *C. retiniana* is found to hybridize with *C. occidentalis* along their zone of contact, and this region was the only area where I found genetic and phenotypic combinations that indicate hybridization between these species. Larvae of both of these species are Abietoideae feeders, so it may be more difficult to maintain reproductive isolation

in zones of contact even though their pheromone attraction is typically different. Further work to determine how *C. retiniana* and *C. occidentalis* maintain their genomic integrity outside of this zone of contact would be of interest.

Incongruence between putatively neutral markers and adaptive traits for identifying species in the spruce budworm complex can be considered from several vantage points. Although adaptive traits (or adaptive genetic markers associated with these traits) and neutral markers may show different patterns of variation, the information from either one is valid, and both are necessary for understanding species and population boundaries. Neutral markers are useful for studying gene flow patterns within and between species at a genomic level, whereas adaptive markers are more suited for studying the gene combinations that contribute to the evolutionary potential or adaptive differences within and between species (Holderegger *et al.*, 2006; Schwartz *et al.*, 2009). Since adaptive genetic markers are presumed to be under selection, it is possible for these markers to be present in one population and absent in another even in the presence of gene flow between populations (Hedrick, 2001; Sperling, 2003; Emelianov *et al.*, 2004; Hey, 2006b; Holderegger *et al.*, 2006).

Studying neutral markers allows us to quantify gene flow between species. Examination of only adaptive markers may suggest that two populations have no gene flow when in fact gene flow is still occurring (Holderegger *et al.*, 2006). If gene flow is still occurring, then it is also possible for adaptive traits that are not tightly linked to species integrity to be passed between species, an example being alleles associated with insecticide resistance or pesticide detoxification (e.g. Pasteur & Raymond, 1996; Labbé *et al.*, 2009). Therefore, knowing from neutral markers that gene flow is possible can alert managers to the possibility of transfer of resistance traits between species that otherwise maintain their genomic integrity.

On the other hand, studying adaptive traits allows us to infer divergence in the midst of gene flow (Hedrick, 2001). As an example, in Finland there are high levels of gene flow between Scots pine (*Pinus sylvestris* L.) populations, as inferred by neutral markers, but the populations show large adaptive genetic

differences (Hedrick, 2001). This is not a false negative, because the neutral markers were correct in identifying continued gene flow, but rather, the true error would have come from not considering adaptive markers to discover the adaptive differences between populations (Hedrick, 2001).

These different processes may account for incongruence between putative neutral markers and adaptive traits among the western species of the spruce budworm complex. Even in the midst of widespread gene flow between species, specific adaptive traits may only be evident in spruce budworm species in situations where the trait provides a fitness benefit. Based on described species differences, adaptive traits that appear to be of particular importance in the spruce budworm complex include host plant association, pheromone production and attraction, length of larval diapause, and morphology. Additional traits of importance may include physiological factors associated with ecogeographical or larval host range, such as egg size and production (Harvey, 1997).

Incongruence between neutral markers and adaptive traits in spruce budworm species provides further evidence in a growing body of literature cautioning against the use of a single locus for species delimitation (e.g. Will & Rubinoff, 2004; Cognato, 2006; Hickerson *et al.*, 2006; Meier *et al.*, 2006; Roe & Sperling, 2007; Schmidt & Sperling, 2008; Shearer & Coffroth, 2008; Lumley & Sperling, 2010). In addition, these findings suggest that even assessing multiple putatively neutral genetic markers appropriate for measuring gene flow will commonly miss economically and ecologically significant species if adaptive traits are not considered. This finding is supported by other researchers who have found that speciation can occur in the midst of gene flow, and that there can be gene flow between species for millions of years after their formation (Rundle *et al.*, 2000; Price & Bouvier, 2002; Bolnick & Near, 2005; Mallet, 2005, 2008). Studying the ecology, behaviour, morphology, and life-history traits of taxa remains important when determining species boundaries. Although consideration of multiple data sets involve increased effort, and may sometimes create more confusion than clarity, it also serves to provide a more biologically realistic interpretation of species. This may be of particular importance for pest species in

which adaptive traits have an impact on their virulence, as well as taxa of conservation concern in which adaptive traits may be the key to their survival (e.g. Bekessy *et al.*, 2003; Emelianov *et al.*, 2004; Turner *et al.*, 2005).

With the rapid developments in population and landscape genomics, it is increasingly feasible to develop markers associated with adaptive traits for natural populations as well as species-specific adaptive genetic markers for species delimitation and identification (Schwartz *et al.*, 2009). Phenotypic expression of some traits is highly associated with environmental conditions (Pfrender *et al.*, 2000), so assaying such genetic markers directly can help to test whether putative adaptive marker variation is actually due to environmental induction of traits.

These findings indicate that although there is gene flow between spruce budworm species, there is also evidence for selective forces acting on the species genomes that are not apparent using neutral markers. Neutral markers used alone frequently fail to distinguish species that can be identified by behaviour, ecology, morphology, and other adaptive traits, but may have speciated in the midst of gene flow. These findings support implementation of an integrative taxonomic approach to species delimitation that is not solely reliant on putatively neutral markers like mtDNA and SSRs.

Table 4-1. Summary of samples, including sample locality, collection ID and date, longitude, latitude, elevation, larval host plant or collection method, collectors, number of individuals (n) used for SSR analysis, number of individuals (n) used for mtDNA analysis, and mtDNA haplotypes with the number of individuals (n) per haplotype given in parentheses.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSR (n)	MtDNA (n)	MtDNA Haplotypes (n)
CAN: AB: East Sousa Creek	LL28	15.vi.2005	-118.495	58.591	393	*Picea glauca*	L. Lumley, M. Maximchuk	10	10	f1 (7), f3 (1), f10 (1), f17 (1)
CAN: AB: Rainbow Lake	LL27	15.vi.2005	-119.404	58.297	532	*Picea glauca*	L. Lumley, M. Maximchuk	10	10	f1 (8), f9 (1), f17 (1)
CAN: AB: La Crete Ferry	LL29	16.vi.2005	-117.113	57.982	318	*Picea glauca*	L. Lumley, et al. [1]	1	1	f1 (1)
CAN: AB: Manning	FS9	20.vi.1991	-117.609	56.915	-	*Picea glauca*	FIDS	0	2	f1 (2)
CAN: AB: EMEND site 2	LL438	20.vi.-25.vii.2007	-118.412	56.750	-	*C. fumiferana* lure	M. Schwarzfeld	1	2	f1 (1), f17 (1)
CAN: AB: Fort McMurray North	LL34	21.vi.2005	-111.407	56.735	281	*Picea glauca*	L. Lumley	25	25	f1 (23), f17 (2)
CAN: AB: Fort McMurray Central	LL33	20.vi.2005	-111.348	56.715	243	*Picea glauca*	L. Lumley	3	3	f1 (2), f2 (1)
CAN: AB: EMEND site 1	LL437	21.vi.-25.vii.2007	-118.216	56.703	-	*C. pinus* lure	M. Schwarzfeld	2	2	o1 (1), o14 (1)
CAN: AB: Fort McMurray South	LL35	21.vi.2005	-111.355	56.686	385	*Picea glauca*	L. Lumley	4	4	f1 (3), f17 (1)
CAN: AB: 120 km S of Fort McMurray	LL36	21.vi.2005	-112.186	55.803	685	*Picea glauca*	L. Lumley	1	1	f1 (1)
CAN: AB: Hythe	LL25	13.vi.2005	-119.455	55.331	757	*Picea glauca*	L. Lumley, et al. [1]	2	2	f1 (2)
CAN: AB: Grande Prairie 1	LL370	23.vi.-6.viii.2007	-118.813	55.087	665	*C. pinus* lure	L. Lumley, J. Doucette	1	2	o89 (1), bβ6 (1)
CAN: AB: Grande Prairie 2	LL371	23.vi.-6.viii.2007	-118.814	55.087	655	*C. fumiferana* lure	L. Lumley, J. Doucette	4	4	f1 (2), f34 (1), bβ (1)
CAN: AB: Lawrence Lake	LL32	17.vi.2005	-113.661	54.993	658	*Picea glauca*	L. Lumley, et al. [1]	1	1	f17 (1)
CAN: AB: Wolf Lake	LL268	2.vii.2006	-110.968	54.707	585	*Picea glauca*	L. Lumley, L. Nolan	5	5	f1 (3), f2 (1), f11 (1)
CAN: AB: Swan Hills 1	LL363	23.vi.-6.viii.2007	-115.411	54.701	1079	*C. pinus* lure	L. Lumley, J. Doucette	1	1	o63 (1)
CAN: AB: Swan Hills 2	LL364	23.vi.-6.viii.2007	-115.411	54.701	1082	*C. fumiferana* lure	L. Lumley, J. Doucette	2	2	f1 (2)
CAN: AB: Between Fort Assiniboine & Swan Hills	LL361	23.vi.-6.viii.2007	-115.174	54.576	877	*C. fumiferana* lure	L. Lumley, J. Doucette	5	5	f1 (3), o2 (1), oβ (1)
CAN: AB: Musreau Lake 2	LL373	23.vi.-6.viii.2007	-118.714	54.564	929	*C. fumiferana* lure	L. Lumley, J. Doucette	1	1	o11 (1)
CAN: AB: Musreau Lake 1	LL372	23.vi.-6.viii.2007	-118.714	54.564	946	*C. pinus* lure	L. Lumley, J. Doucette	1	1	bβ (1)
CAN: AB: 42 S of Swan Hills	LL365b	23.vi.-6.viii.2007	-115.643	54.404	1122	*C. fumiferana* lure	L. Lumley, J. Doucette	5	5	f1 (4), f5 (1)
CAN: AB: Fort Assiniboine 2	LL360	23.vi.-6.viii.2007	-114.810	54.303	653	*C. fumiferana* lure	L. Lumley, J. Doucette	6	6	f1 (1), f27 (1), o1 (1), o2 (1), oβ (1), oβ12 (1)
CAN: AB: Fort Assiniboine	LL359	23.vi.-6.viii.2007	-114.811	54.302	654	*C. pinus* lure	L. Lumley, J. Doucette	9	10	p1 (9), p11 (1)
CAN: AB: Virginia Hills Rd	LL366	23.vi.-6.viii.2007	-116.169	54.288	858	*C. fumiferana* lure	L. Lumley, J. Doucette	5	5	f1 (3), f5 (1), f29 (1)
CAN: AB: Pine Ridge Nursery	FS10	26.vi.1997	-112.500	54.283	-	*Pinus banksiana*	J. Volney	0	2	p1 (1), p9 (1)
CAN: AB: Moose Lake	LL780	1.vii.2008	-111.005	54.246	560	*Pinus banksiana*	L. Lumley	5	5	p1 (5)
CAN: AB: 50 km S of Musreau Lake 2	LL375	23.vi.-6.viii.2007	-118.649	54.207	1252	*C. pinus* lure	L. Lumley, J. Doucette	4	4	o1 (2), o46 (1), o81 (1)
CAN: AB: 50 km S of Musreau Lake 1	LL374	23.vi.-6.viii.2007	-118.649	54.206	1251	*C. fumiferana* lure	L. Lumley, J. Doucette	1	1	o1 (1)
CAN: AB: Clyde	LL353	17-25.vii.2006	-113.500	54.154	660	*C. pinus* lure	L. Lumley	5	5	p1 (5)
CAN: AB: Bellis West	LL349	15-25.vii.2006	-112.165	54.120	659	*C. pinus* lure	L. Lumley, L. Nolan	5	5	p1 (2), p4 (1), p6 (1), p7 (1)
CAN: AB: Bellis West L2	LL781	1.vii.2008	-112.169	54.117	654	*Pinus banksiana*	L. Lumley	3	3	p1 (2), p7 (1)
CAN: AB: Bellis West L1	LL779	1.vii.2008	-112.190	54.112	638	*Pinus banksiana*	L. Lumley	1	1	p8 (1)
CAN: AB: Simonette River Trap 1	LL119	5.vii.-5.xi.2005	-118.480	54.105	-	*C. fumiferana* lure	ASRD	2	2	o1 (2)
CAN: AB: Simonette River Trap 2	LL120	5.vii.-5.xi.2005	-118.480	54.105	-	*C. fumiferana* lure	ASRD	3	3	o1 (1), o2 (2)
CAN: AB: Bellis South	LL348	15-25.vii.2006	-112.128	54.095	664	*C. pinus* lure	L. Lumley, L. Nolan	5	5	p1 (4), p3 (1)
CAN: AB: Smoky Lake Sands E	LL351	15-25.vii.2006	-112.285	54.065	612	*C. pinus* lure	L. Lumley, L. Nolan	5	5	p1 (4), p4 (1)
CAN: AB: Smoky Lake Sands W	LL350	15-25.vii.2006	-112.318	54.050	617	*C. pinus* lure	L. Lumley, L. Nolan	5	5	p1 (3), p5 (1), p8 (1)
CAN: AB: Opal 2	LL355	17-25.vii.2006	-113.221	54.037	638	*C. pinus* lure	L. Lumley	5	5	o1 (1), p1 (4)
CAN: AB: Opal 1	LL354	17-25.vii.2006	-113.275	54.010	646	*C. pinus* lure	L. Lumley	5	5	p1 (5)
CAN: AB: Redwater 2	LL357	17-25.vii.2006	-112.952	53.943	631	*C. pinus* lure	L. Lumley	5	5	p1 (2), p4 (2), p5 (1)
CAN: AB: Bellis Southeast	LL352	15-25.vii.2006	-111.982	53.940	640	*C. pinus* lure	L. Lumley, L. Nolan	5	5	p1 (4), p3 (1)
CAN: AB: Redwater 1	LL356	17-25.vii.2006	-112.952	53.937	628	*C. pinus* lure	L. Lumley	3	5	o1 (1), p1 (3), p4 (1)
CAN: AB: Muskeg River 1	LL376	24.vi.-6.viii.2007	-118.822	53.925	1157	*C. fumiferana* lure	L. Lumley, J. Doucette	5	5	o1 (2), o2 (2), o24 (1)
CAN: AB: Muskeg River 2	LL377	24.vi.-6.viii.2007	-118.823	53.925	1156	*C. pinus* lure	L. Lumley, J. Doucette	1	1	o1 (1)
CAN: AB: Cote Creek	LL113	5.vii.-5.xi.2005	-119.913	53.912	-	*C. fumiferana* lure	ASRD	2	2	o1 (1), o2 (1)
CAN: AB: Lower Sheep Creek	LL112	5.vii.-5.xi.2005	-119.548	53.870	-	*C. fumiferana* lure	ASRD	3	3	o1 (2), o2 (1)

Table 4-1, cont.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSRs (n)	MtDNA (n)	MtDNA Haplotypes (n)
CAN: AB: Whitney Lakes	LL358	17-25.vii.2006	-110.535	53.828	607	*C. pinus* lure	L. Lumley	5	5	p1 (4), bβ9 (1)
CAN: AB: Muddy Water River Trap 1	LL114	5.vii.-5.xi.2005	-119.688	53.811	-	*C. fumiferana* lure	ASRD	2	2	o1 (1), o2 (1)
CAN: AB: Muddy Water River Trap 2	LL115	5.vii.-5.xi.2005	-119.688	53.811	-	*C. fumiferana* lure	ASRD	3	3	o1 (2), b1 (1)
CAN: AB: Casket Lake	LL111	5.vii.-5.xi.2005	-119.926	53.783	-	*C. fumiferana* lure	ASRD	2	2	o1 (1), o2 (1)
CAN: AB: Hendrickson Creek Trap 1	LL117	5.vii.-5.xi.2005	-118.351	53.782	-	*C. fumiferana* lure	ASRD	3	3	o1 (1), o2 (1), b1 (1)
CAN: AB: Hendrickson Creek Trap 2	LL118	5.vii.-5.xi.2005	-118.351	53.782	-	*C. fumiferana* lure	ASRD	2	2	o2 (2)
CAN: AB: Athabasca River Trap 1	LL121	5.vii.-5.xi.2005	-117.157	53.703	-	*Picea glauca*	ASRD	2	3	f1 (1), f31 (1), o1 (1)
CAN: AB: Athabasca River Trap 2	LL122	5.vii.-5.xi.2005	-117.157	53.703	-	*Picea glauca*	ASRD	4	4	f13 (1), o1 (2), o2 (1)
CAN: AB: Jackpine River / Spider Creek Trap 1	LL109	5.vii.-5.xi.2005	-119.786	53.684	-	*C. fumiferana* lure	ASRD	1	1	o2 (1)
CAN: AB: Jackpine River / Spider Creek Trap 2	LL110	5.vii.-5.xi.2005	-119.786	53.684	-	*C. fumiferana* lure	ASRD	3	3	o1, (1), o2 (1), b1 (1)
CAN: AB: Moon Creek	LL116	5.vii.-5.xi.2005	-118.440	53.650	-	*C. fumiferana* lure	ASRD	3	4	o1 (2), o2 (1), f1 (1)
CAN: AB: S of Little Berland River 1	LL378	24.vi.-7.viii.2007	-118.141	53.630	1431	*C. fumiferana* lure	L. Lumley, J. Doucette	6	6	o1 (1), o2 (5)
CAN: AB: S of Little Berland River 2	LL379	24.vi.-7.viii.2007	-118.142	53.630	1423	*C. pinus* lure	L. Lumley, J. Doucette	1	1	o1 (1)
CAN: AB: S of Nojack	LL386	24.vi.-7.viii.2007	-115.617	53.608	838	*C. pinus* lure	L. Lumley, J. Doucette	4	4	bβ6 (2), bβ7 (2)
CAN: AB: Hornbeck Creek	LL17	11.vi.2005	-116.685	53.574	934	*Picea glauca*	L. Lumley, et al. [1]	2	2	f1 (2)
CAN: AB: W of Bickerdike Rd 1	LL384	24.vi.-7.viii.2007	-116.720	53.567	977	*C. pinus* lure	L. Lumley, J. Doucette	3	3	bβ (3)
CAN: AB: W of Bickerdike Rd 2	LL385	24.vi.-7.viii.2007	-116.723	53.566	999	*C. fumiferana* lure	L. Lumley, J. Doucette	5	5	f1 (2), o2 (1), o31 (1), b1 (1)
CAN: AB: Edmonton	LL149	20.v.2006	-113.470	53.550	-	*Picea glauca*	L. Lumley	1	1	f1 (1)
CAN: AB: S of William Switzer 1	LL380	24.vi.-7.viii.2007	-117.755	53.428	1353	*C. fumiferana* lure	L. Lumley, J. Doucette	10	10	o1 (1), o2 (5), o5 (1), o37 (1), o47 (1), b1 (1)
CAN: AB: S of William Switzer 2	LL381	24.vi.-7.viii.2007	-117.754	53.428	1362	*C. pinus* lure	L. Lumley, J. Doucette	2	2	o1 (2)
CAN: AB: Jackpine River	LL107	5.vii.-5.xi.2005	-119.554	53.395	-	*C. fumiferana* lure	ASRD	3	3	o1 (2), b1 (1)
CAN: AB: 7 km N of Cynthia	LL16	11.vi.2005	-115.427	53.340	964	*Picea glauca*	L. Lumley, et al. [1]	3	3	f1 (3)
CAN: AB: Lund Creek Trap 2	LL123	5.vii.-5.xi.2005	-116.563	52.983	-	*C. fumiferana* lure	ASRD	2	2	o2 (1), o33 (1)
CAN: AB: Brown Creek	LL99	27.vii.2005	-116.363	52.764	1262	*Pinus contorta*	L. Lumley, A. Roe	1	1	o1 (1)
CAN: AB: Jasper NP, Honeymoon Lake	LL304	12-24.vii.2006	-117.724	52.577	1294	*C. fumiferana* lure	L. Lumley	5	5	o2 (1), b1 (4)
CAN: AB: Jasper NP, Honeymoon Lake	LL305	12-24.vii.2006	-117.723	52.576	1289	*C. pinus* lure	L. Lumley	1	1	bβ (1)
CAN: AB: 15 km E of Nordegg 1	LL775	30.vi.2008	-115.841	52.488	1323	*C. pinus* lure	L. Lumley	3	3	o1 (3)
CAN: AB: 15 km E of Nordegg 2	LL776	30.vi.2008	-115.842	52.488	1287	*C. fumiferana* lure	L. Lumley	10	10	o2 (4), b1 (6)
CAN: AB: Crimson Lake	LL15	11.vi.2005	-115.028	52.439	1038	*Picea glauca*	L. Lumley, et al. [1]	2	2	f1 (2)
CAN: AB: 60 km E of Nordegg 1	LL777	30.vi.2008	-115.229	52.421	1064	*C. pinus* lure	L. Lumley	1	1	b1 (1)
CAN: AB: 60 km E of Nordegg 2	LL778	30.vi.2008	-115.229	52.420	1051	*C. fumiferana* lure	L. Lumley	10	10	f1 (7), f30 (1), b1 (2)
CAN: AB: Jasper / Banff NP border	LL303	12-24.vii.2006	-117.159	52.215	2054	*C. fumiferana* lure	L. Lumley	5	5	o1 (1), o2 (1), o11 (1), b1 (2)
CAN: AB: 50 km W of Nordegg	LL774	30.vi.2008	-116.472	52.166	1332	*Pinus contorta*	L. Lumley	3	3	bβ (3)
CAN: AB: Thompson Creek	LL13	10.vi.2005	-116.628	52.012	1391	*Picea glauca*	L. Lumley, et al. [1]	1	1	b1 (1)
CAN: AB: Banff NP, Saskatchwan Crossing	LL302	12-24.vii.2006	-116.735	51.973	1457	*C. pinus* lure	L. Lumley	9	9	o1 (3), o2 (5), b2 (1)
CAN: AB: Red Lodge	FS11	12.vi.1992	-114.240	51.943	956	*Picea glauca*	F. Sperling, J. Volney	0	3	f1 (2), b1 (1)
CAN: AB: Red Lodge	LL79	13.vii.2005	-114.240	51.943	956	*Picea glauca*	L. Lumley, A. Roe	1	1	f1 (1)
CAN: AB: Red Lodge	LL85	23.vii.2005	-114.240	51.943	956	UV light	L. Lumley	9	9	f1 (9)
CAN: AB: W of Sundre	LL11	9.vi.2005	-115.053	51.888	1259	*Picea glauca*	L. Lumley, et al. [1]	1	1	f1 (1)
CAN: AB: Banff NP, Mosquito Creek	LL300	12-24.vii.2006	-116.327	51.627	1814	*C. fumiferana* lure	L. Lumley	10	10	o2 (4), o69 (1), b1 (5)
CAN: AB: Banff NP, Smith Lake	LL294	12-24.vii.2006	-115.933	51.261	1441	*C. pinus* lure	L. Lumley	5	5	o2 (1), b2 (4)
CAN: AB: West Spray Lakes	LL73	12.vii.2005	-115.372	50.991	1678	*Picea engelmannii*	L. Lumley, A. Roe	4	4	o60 (1), b1 (3)
CAN: AB: Bragg Creek	LL10	9.vi.2005	-114.576	50.945	1313	*Picea glauca*	L. Lumley, et al. [1]	11	11	f1 (9), f26 (1), o1 (1)
CAN: AB: Wedge Pond	LL76	12.vii.2005	-115.147	50.874	1521	*Picea engelmannii*	L. Lumley, A. Roe	2	2	o2 (1), b1 (1)
CAN: AB: Buller Mountain	LL74	12.vii.2005	-115.354	50.868	1765	*Picea engelmannii*	L. Lumley, A. Roe	6	6	o11 (1), b1 (5)
CAN: AB: Buller Mountain	LL74	12.vii.2005	-115.354	50.868	1765	*Pseudo. menziesii*	L. Lumley, A. Roe	1	1	o2 (1)
CAN: AB: Sawmill Picnic Area	LL75	12.vii.2005	-115.245	50.749	1799	*Picea engelmannii*	L. Lumley, A. Roe	3	3	o2 (2), b1 (1)

Table 4-1, cont.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSRs (n)	MtDNA (n)	MtDNA Haplotypes (n)
CAN: AB: Little Highwood Pass	LL291	12.vii.2006	-115.066	50.643	1860	*Picea engelmannii*	L. Lumley	1	1	b1 (1)
CAN: AB: Little Highwood Pass	LL291	23-24.vii.2006	-115.066	50.643	1860	*C. fumiferana* lure	L. Lumley	5	5	o1 (1), o13 (1), b1 (3)
CAN: AB: Mount Sarrail	LL41	5.vii.2005	-115.122	50.618	1692	*Picea engelmannii*	L. Lumley, A. Roe	6	6	o1 (2), b1 (4)
CAN: AB: Mount Sarrail	LL41	5.vii.2005	-115.122	50.618	1692	*Pseudo. menziesii*	L. Lumley, A. Roe	1	1	o1 (1)
CAN: AB: Forestry Trunk Rd, 13 km N of Rd 532	LL42	5.vii.2005	-114.592	50.236	1786	*Picea glauca*	L. Lumley, A. Roe	1	1	b1 (1)
CAN: AB: Skyline Rd, 1 km S of Rd 520	LL435	30.vii.2007	-114.088	49.972	1694	*C. pinus* lure	L. Lumley, et al. [2]	5	5	o2 (1), o13 (1), b1 (3)
CAN: AB: Skyline Rd, 1 km S of Rd 520	LL50	7.vii.2005	-114.087	49.971	1675	*Pseudo. menziesii*	L. Lumley, A. Roe	5	5	o2 (3), o11 (1), oβ (1)
CAN: AB: Skyline Rd, 1 km S of Rd 520	LL764	29.vi.2008	-114.087	49.970	1707	*Pinus contorta*	L. Lumley	2	2	o1 (1), o2 (1)
CAN: AB: Porcupine Hills, Rd 520 E of Hwy 22	LL7	9.vi.2005	-114.115	49.968	1459	*Picea glauca*	L. Lumley, et al. [1]	5	5	o1 (1), o2 (2), o35 (1), oβ (1)
CAN: AB: Dutch Creek	LL39	5.vii.2005	-114.397	49.900	1399	*Picea glauca*	L. Lumley, A. Roe	1	1	o11 (1)
CAN: AB: Rd 517, 7 km E of Forestry Trunk Rd	LL47	6.vii.2005	-114.347	49.871	1425	*Pseudo. menziesii*	L. Lumley, A. Roe	5	5	o2 (3), o44 (1), o51 (1)
CAN: AB: Rd 517, 5 km E of Forestry Trunk Rd	LL46	6.vii.2005	-114.360	49.867	1402	*Picea glauca*	L. Lumley, A. Roe	1	1	o2 (1)
CAN: AB: Forestry Trunk Rd, 1 km S of Rd 517	LL49	6.vii.2005	-114.403	49.851	1439	*Picea glauca*	L. Lumley, A. Roe	1	1	o2 (1)
CAN: AB: Rd 517, 11 km E of Forestry Trunk Rd	LL48	6.vii.2005	-114.264	49.845	1398	*Pseudo. menziesii*	L. Lumley, A. Roe	5	5	o1 (1), o2 (1), o11 (1), o34 (1), o44 (1)
CAN: AB: Porcupine Hills, Beaver Creek	LL51	7.vii.2005	-113.948	49.804	1377	*Pseudo. menziesii*	L. Lumley, A. Roe	25	25	o1 (2), o2 (11), o11 (1), o20 (2), o28 (1), o30 (1), o36 (1), o40 (1), o44 (1), o50 (1), oβ (3)
CAN: AB: Cypress Hills, Firerock CG	LL6	8.vi.2005	-110.321	49.657	1293	*Picea glauca*	L. Lumley, et al. [1]	10	10	f1 (4), f7 (1), f15 (1), f17 (1), o2 (1), o11 (1), o18 (1)
CAN: AB: Hillcrest Mine Rd	LL58	10.vii.2005	-114.324	49.559	1223	*Pseudo. menziesii*	L. Lumley, A. Roe	7	7	o1 (1), o2 (4), o5 (2)
CAN: AB: Waterton Lake NP Coll 2	LL53	8.vii.2005	-113.791	49.066	1618	*Pseudo. menziesii*	L. Lumley, A. Roe	4	4	o2 (1), o13 (1), o25 (1), b1 (1)
CAN: AB: Waterton Lake NP Coll 1	LL52	8.vii.2005	-113.737	49.059	1546	*Picea glauca*	L. Lumley, A. Roe	1	1	o5 (1)
CAN: AB: Waterton Lake NP Coll 3	LL54	8.vii.2005	-113.926	49.059	1457	*Pseudo. menziesii*	L. Lumley, A. Roe	1	1	o2 (1)
CAN: AB: Cypress Hills	FS12	11.vi.1992	-110.321	49.657	-	*Picea glauca*	F. Sperling, J. Volney	0	4	f1 (2), f4 (1), o2 (1)
CAN: BC: N of USA / CAN border crossing	LL190	15.vi.2006	-135.196	59.578	653	*Picea sitchensis*	L. Lumley, et al. [1]	3	3	o1 (3)
CAN: BC: Kinaskan Lake	LL173	9.vi.2006	-130.252	57.343	803	*Abies lasiocarpa*	L. Lumley, et al. [1]	2	2	o1 (2)
CAN: BC: Hodder Lake	LL171	9.vi.2006	-129.780	56.726	621	*Abies lasiocarpa*	L. Lumley, et al. [1]	5	5	o1 (5)
CAN: BC: Daft Creek	LL170	9.vi.2006	-129.435	56.500	464	*Abies lasiocarpa*	L. Lumley, et al. [1]	2	2	o1 (2)
CAN: BC: Morrisey Creek	FS1	9.vii.1991	-124.550	56.333	-	*Abies lasiocarpa*	FIDS	0	3	b1 (2), bβ (1)
CAN: BC: Mesiadin Junction	LL169	9.vi.2006	-129.348	56.105	304	*Picea glauca*	L. Lumley, et al. [1]	1	1	o81 (1)
CAN: BC: Stewart	LL168	9.vi.2006	-129.997	55.935	30	*Picea sitchensis*	L. Lumley, et al. [1]	5	5	o1 (3), b1 (2)
CAN: BC: New Aiyansh	LL160	8.vi.2006	-128.722	55.543	197	*Picea glauca*	L. Lumley, et al. [1]	3	3	b1 (3)
CAN: BC: Kincolith	LL163	8.vi.2006	-129.839	55.005	21	*Picea sitchensis*	L. Lumley, et al. [1]	4	4	o1 (4)
CAN: BC: Purden Lake	LL151	6.vi.2006	-121.954	53.917	767	*Picea glauca*	L. Lumley, et al. [1]	2	2	o1 (1), b1 (1)
CAN: BC: Purden Lake	LL151	6.vi.2006	-121.954	53.917	767	*Pseudo. menziesii*	L. Lumley, et al. [1]	3	3	o62 (1), b1 (2)
CAN: BC: Slim Creek	LL266	28.vi.2006	-121.142	53.726	678	*Picea glauca*	L. Lumley, et al. [1]	1	1	b1 (1)
CAN: BC: McBride	LL791	5.viii.2008	-120.167	53.300	-	Butterfly net	L. Lumley, J. Doucette	11	11	b1 (8), o1 (1), o2 (1), o93 (1)
CAN: BC: McBride	FS2	4.vi.1994	-120.167	53.300	-	*Abies lasiocarpa*	FIDS	0	2	b1 (1), bβ (1)
CAN: BC: Yoho NP, Field	LL299	12-24.vii.2006	-116.477	51.406	1248	*C. fumiferana* lure	L. Lumley	5	5	o2 (2), o62 (1), o92 (1), b1 (1)
CAN: BC: Kootenay / Banff boundary on Hwy 93	LL72	11.vii.2005	-116.054	51.226	1640	*Picea engelmannii*	L. Lumley, A. Roe	2	2	b1 (2)
CAN: BC: Golden	LL298	12-24.vii.2006	-116.828	51.171	866	*C. fumiferana* lure	L. Lumley	5	5	o2 (1), o73 (1), o91 (1), b1 (2)
CAN: BC: Numa Falls	FS3	12.vi.1992	-116.126	51.131	-	*Picea engelmannii*	F. Sperling, J. Volney	0	3	b1 (1), b2 (1), b3 (1)
CAN: BC: Kootenay N.P., unmarked picnic site	LL71	11.vii.2005	-115.972	50.950	1270	*Picea engelmannii*	L. Lumley, A. Roe	8	8	o1 (1), o70 (1), b1 (6)
CAN: BC: Kootenay NP, Dolly Varden	LL295	12-24.vii.2006	-116.015	50.826	1188	*C. fumiferana* lure	L. Lumley	5	5	o1 (1), o2 (1), o80 (1), b1 (2)
CAN: BC: Edgewater	LL297	12-24.vii.2006	-116.196	50.767	891	*C. fumiferana* lure	L. Lumley	5	5	o1 (2), o2 (1), b1 (2)
CAN: BC: Kootenay NP, Truck brake-check	LL296	12-24.vii.2006	-115.946	50.681	1488	*C. fumiferana* lure	L. Lumley	5	5	o2 (2), b1 (2), o43 (1)
CAN: BC: Monte Creek	FS4	20.vi.1991	-119.949	50.646	-	*Pseudo. menziesii*	FIDS	10	2	o1 (1), o3 (1)
CAN: BC: Wilmer	LL68	11.vii.2005	-116.073	50.557	917	*Pseudo. menziesii*	L. Lumley, A. Roe	3	3	o1 (2), o2 (1)
CAN: BC: Invermere	LL67	11.vii.2005	-116.059	50.514	969	*Pseudo. menziesii*	L. Lumley, A. Roe	1	1	b1 (1)

Table 4-1, cont.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSRs (n)	MtDNA (n)	MtDNA Haplotypes (n)
CAN: BC: Vernon	LL104	9.viii.2005	-119.269	50.208	-	UV light	A. Roe	5	5	o1 (1), o2 (1), o49 (1), o61 (1), b1 (1)
CAN: BC: Canal Flats	LL66	11.vii.2005	-115.810	50.164	871	UV light	L. Lumley, A. Roe	1	1	o1 (1)
CAN: BC: Whiteswan Lake 1	LL772	29.vi.2008	-115.771	50.118	849	*C. pinus* lure	L. Lumley	3	3	o1 (1), o41 (1), b1 (1)
CAN: BC: Whiteswan Lake 2	LL773	29.vi.2008	-115.770	50.117	839	*C. fumiferana* lure	L. Lumley	9	10	o1 (1), o2 (1), o74 (1), b1 (7)
CAN: BC: Winfield	FS5	21.vi.1991	-119.310	50.080	-	*Pseudo. menziesii*	FIDS	10	3	o2 (1), o4 (1), o9 (1)
CAN: BC: Sparwood	LL60	10.vii.2005	-114.850	49.725	1148	*Pseudo. menziesii*	L. Lumley, A. Roe	5	5	o2 (3), b1 (2)
CAN: BC: Fort Steele	LL770	29.vi.2008	-115.666	49.668	823	*C. pinus* lure	L. Lumley	1	1	o2 (1)
CAN: BC: Fort Steele	LL64	10.vii.2005	-115.566	49.649	828	*Pseudo. menziesii*	L. Lumley, A. Roe	1	1	o2 (1)
CAN: BC: Hosmer	LL765	29.vi.2008	-114.950	49.606	1065	*Pinus contorta*	L. Lumley	7	7	o1 (5), o72 (1), b1 (1)
CAN: BC: Fernie	LL61	10.vii.2005	-115.061	49.446	970	*Picea engelmannii x glauca*	L. Lumley, A. Roe	5	5	o2 (4), o5 (1)
CAN: BC: Fernie West 2	LL767	29.vi.2008	-115.013	49.345	975	*C. pinus* lure	L. Lumley	7	7	o1 (1), o2 (4), b1 (1), o11 (1)
CAN: BC: Fernie West 1	LL766	29.vi.2008	-115.014	49.345	963	*C. fumiferana* lure	L. Lumley	10	10	o1 (2), o2 (6), o28 (1), oβ (1)
CAN: BC: Elko 1	LL768	29.vi.2008	-115.154	49.292	874	*C. fumiferana* lure	L. Lumley	10	8	o2 (7), o52 (1)
CAN: BC: Elko 2	LL769	29.vi.2008	-115.155	49.292	878	*C. pinus* lure	L. Lumley	5	5	o1 (1), o2 (2), o5 (1), o28 (1)
CAN: BC: Greenwood	FS6	15.vi.1992	-118.683	49.083	-	*Pseudo. menziesii*	FIDS	11	5	o1 (1), o2 (1), o7 (1), o8 (1), o9 (1)
CAN: BC: Bridesville	FS7	16.vi.1992	-119.115	49.072	-	*Pseudo. menziesii*	FIDS	9	3	o5 (1), o6 (1), oβ (1)
CAN: MB: Snow Lake	LL509	Summer 2007	-99.949	54.839	-	*C. fumiferana* lure	MCFB	5	5	f1 (4), f17 (1)
CAN: MB: Moose Lake Road	LL516	Summer 2007	-100.879	53.851	-	*C. pinus* lure	MCFB	5	5	p1 (5)
CAN: MB: St. Martin	LL515	Summer 2007	-98.810	51.895	-	*C. pinus* lure	MCFB	5	5	p1 (5)
CAN: MB: Pelican Lake	LL501	Summer 2007	-101.151	51.668	-	*C. fumiferana* lure	MCFB	5	5	f1 (4), f31 (1)
CAN: MB: O'Hanly River	LL498	Summer 2007	-96.217	50.791	-	*C. fumiferana* lure	MCFB	5	5	f1 (3), f17 (1), f33 (1)
CAN: MB: Carberry	FS13	17.vi.1991	-99.359	49.869	-	*Picea glauca*	FIDS	10	2	f1 (2)
CAN: MB: Richer	LL520	Summer 2007	-96.201	49.630	-	*C. pinus* lure	MCFB	5	5	p1 (5)
CAN: NB: Mt. Carleton	FS14	20.vi.1991	-66.100	48.167	-	*Picea glauca*	FIDS	5	0	
CAN: NB: Edmunston	LL466	14.vi.-5.ix.2007	-68.483	47.447	-	*C. fumiferana* lure	NBNR	5	5	f1 (5)
CAN: NB: Holmes Lake	FS15	17.vi.1991	-66.617	46.950	-	*Abies balsamea*	FIDS	0	2	f1 (1), f3 (1)
CAN: NB: Dunphy	LL441	29.vi.-4.ix.2007	-65.756	46.675	-	*C. pinus* lure	NBNR	5	5	p1 (4), p12 (1)
CAN: NB: Hartland	LL470	9.vi.-24.viii.2007	-67.516	46.469	-	*C. fumiferana* lure	NBNR	5	5	f1 (5)
CAN: NB: Sussex	LL444	28.vi.-13.ix.2007	-65.581	45.822	-	*C. pinus* lure	NBNR	4	5	p1 (2), p4 (1), p8 (1), p13 (1)
CAN: NB: Area K1	LL474	31.v.-24.viii.2007	-67.179	45.755	-	*C. fumiferana* lure	NBNR	5	5	f1 (5)
CAN: NB: Area Q1	LL443	22.vi.-10.ix.2007	-66.788	45.391	-	*C. pinus* lure	NBNR	5	5	p1 (4), p10 (1)
CAN: NFLD: Blanch River	FS21	29.vii.1991	-57.970	48.941	-	*Abies balsamea*	FIDS	9	2	f1 (2)
CAN: NS: Lake Rosignal	LL789	25.ii.2008	-65.107	44.209	-	*Pinus strobus*	NSDNR	4	4	p1 (2), p12 (2)
CAN: ON: Balmer	LL713	Summer 2007	-93.688	51.356	-	*C. pinus* lure	OMNR	5	5	p1 (5)
CAN: ON: Kenora	LL582	Summer 2007	-94.293	49.807	-	*C. fumiferana* lure	OMNR	5	4	f1 (4)
CAN: ON: Ignace	FS16	14.vi.1991	-91.667	49.417	-	*Picea glauca*	FIDS	7	2	f1 (1), f2 (1)
CAN: ON: Wawa	LL577	Summer 2007	-84.772	49.289	-	*C. fumiferana* lure	OMNR	5	5	f1 (5)
CAN: ON: Timmins	LL676	Summer 2007	-81.853	47.467	-	*C. pinus* lure	OMNR	5	4	p1 (4)
CAN: ON: Pembina	LL667	Summer 2007	-77.580	45.657	-	*C. pinus* lure	OMNR	4	3	p1 (3)
CAN: ON: Parry Sound	FS17	20.vi.1991	-80.036	45.354	-	*Pinus banksiana*	FIDS	0	5	p1 (3), p2 (1), r2 (1)
CAN: ON: Peterborough	LL599	Summer 2007	-78.419	44.269	-	*C. fumiferana* lure	OMNR	5	5	f1 (4), f28 (1)
CAN: QC: Lac Labrie	LL547	Summer 2007	-66.665	50.183	76	*C. fumiferana* lure	QMNRW	5	5	f1 (3), f11 (1), f13 (1)
CAN: QC: Bleutiere Normandin	LL551	Summer 2007	-72.562	48.776	152	*C. pinus* lure	QMNRW	4	5	p1 (5)
CAN: QC: Causapscal	FS18	3.vii.1991	-67.232	48.372	-	*Picea glauca*	FIDS	5	0	
CAN: QC: Nedelec	LL549	Summer 2007	-79.389	47.663	274	*C. pinus* lure	QMNRW	5	5	p1 (5)
CAN: QC: Lac Ravalet	LL544	Summer 2007	-79.149	46.841	259	*C. fumiferana* lure	QMNRW	5	5	f1 (5)
CAN: QC: Lac Bouchard	LL543	Summer 2007	-72.807	46.758	150	*C. fumiferana* lure	QMNRW	5	5	f1 (5)

Table 4-1, cont.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSRs (n)	MtDNA (n)	MtDNA Haplotypes (n)
CAN: QC: Ste.Agathe	FS19	Summer 1992	-74.276	46.060	-	UV light	J.F. Landry	0	1	r1 (1)
CAN: QC: Fort-Coulonge	LL550	Summer 2007	-76.715	45.830	107	*C. pinus* lure	QMNRW	5	5	p1 (5)
CAN: SK: Deschambault Village	LL103	4.vii.2005	-103.3	55.6	-	*Picea glauca*	SK Environment	2	2	f1 (2)
CAN: SK: North Vivian Lake	LL140	Summer 2005	-108.90778	54.422778	-	*C. pinus* lure	SK Environment	1	3	p1 (2), o2 (1)
CAN: SK: Hwy 4 / Hwy 224 Junction	LL141	Summer 2005	-108.85556	54.421667	-	*C. pinus* lure	SK Environment	1	2	p1 (2)
CAN: SK: Worthington Lake	LL133	Summer 2005	-109.59075	53.981181	-	*C. pinus* lure	SK Environment	2	2	p1 (2)
CAN: SK: Worthington Lake	LL132	Summer 2005	-109.59076	53.98118		*C. pinus* lure	SK Environment	1	1	o1 (1)
CAN: SK: Peck Lake	LL131	Summer 2005	-109.56947	53.898872	-	*C. pinus* lure	SK Environment	3	2	o2 (2)
CAN: SK: Miko Lake	LL142	Summer 2005	-107.72222	53.894167	-	*C. pinus* lure	SK Environment	0	2	p1 (2)
CAN: SK: PANP (South)	LL144	Summer 2005	-106.09639	53.77606	-	*C. pinus* lure	SK Environment	0	2	p1 (2)
CAN: SK: Pasquia-Porcupine FMA	LL795	Summer 2008	-102.48047	53.690201	-	*C. fumiferana* lure	SK Environment	5	5	f1 (5)
CAN: SK: Hwy 4 Comm Twr	LL128	Summer 2005	-108.3625	53.645278	-	*C. pinus* lure	SK Environment	1	1	o53 (1)
CAN: SK: Hwy 4 Comm Twr	LL129	Summer 2005	-108.3625	53.645278	-	*C. pinus* lure	SK Environment	1	1	o2 (1)
CAN: SK: Moose County Store	LL130	Summer 2005	-108.36486	53.622461	-	*C. pinus* lure	SK Environment	0	1	p1 (1)
CAN: SK: Elk House Tower	LL136	Summer 2005	-105.09058	53.352892	-	*C. pinus* lure	SK Environment	2	3	p1 (3)
CAN: SK: W of Nipawin	LL137	Summer 2005	-104.31919	53.347883	-	*C. pinus* lure	SK Environment	3	3	p1 (3)
CAN: SK: Canwood NW	LL134	Summer 2005	-106.54564	53.340828	-	*C. pinus* lure	SK Environment	1	1	p1 (1)
CAN: SK: Canwood SE	LL135	Summer 2005	-106.53153	53.328439	-	*C. pinus* lure	SK Environment	1	0	
CAN: SK: Holbein	LL139	Summer 2005	-106.1949	53.2303	-	*C. pinus* lure	SK Environment	1	2	p4(1), p10 (1)
CAN: YT: Top of the World Hwy, Km 32	LL237	23.vi.2006	-139.877	64.174	1100	*Picea glauca*	L. Lumley, et al. [1]	5	5	o1 (1), bβ (4)
CAN: YT: Top of the World Hwy, Km 86	LL236	22.vi.2006	-140.694	64.116	1037	*Picea glauca*	L. Lumley, et al. [1]	5	5	f1 (1), o2 (1), bβ (3)
CAN: YT: Klondike Hwy, Km 319	LL240	25.vi.2006	-136.420	62.356	527	*Picea glauca*	L. Lumley, et al. [1]	1	1	f1 (1)
CAN: YT: White River	LL202	17.vi.2006	-140.537	61.985	743	*Picea glauca*	L. Lumley, et al. [1]	2	2	o1 (2)
CAN: YT: Pine Valley Lodge	LL201	16.vi.2006	-140.045	61.806	827	*Picea glauca*	L. Lumley, et al. [1]	5	5	o1 (2), o2 (2), o48 (1)
CAN: YT: Destruction Bay	LL200	16.vi.2006	-138.813	61.256	829	*Picea glauca*	L. Lumley, et al. [1]	5	5	o1 (3), o54 (1), b1 (1)
CAN: YT: Spruce Beetle Trail	LL199	16.vi.2006	-137.768	60.840	988	*Picea glauca*	L. Lumley, et al. [1]	1	1	o1 (1)
CAN: YT: Campbell Hwy	LL248	26.vi.2006	-129.173	60.612	690	*Picea glauca*	L. Lumley, et al. [1]	2	2	bβ (2)
CAN: YT: Teslin	LL183	11.vi.2006	-132.679	60.153	820	*Picea glauca*	L. Lumley, et al. [1]	2	2	o42 (1), b2 (1)
USA: AK: Hwy 3 mi 341, SW of Fairbanks	LL228	20.vi.2006	-148.237	64.785	439	*Picea glauca*	L. Lumley, et al. [1]	5	5	f1 (4), f25 (1)
USA: AK: Fairbanks	FS25	1-22.vii.1992	-148.237	64.785	-	*C. orae* lure	USFS	0	3	o1 (2), b1 (1)
USA: AK: Fairbanks	FS26	1-22.vii.1992	-148.237	64.785	-	*C. fumiferana* lure	USFS	0	3	f1 (3)
USA: AK: Hwy 3 Mile 319	LL227	20.vi.2006	-148.832	64.693	321	*Picea glauca*	L. Lumley, et al. [1]	5	5	f1 (4), f33 (1)
USA: AK: Hwy 2 mi 37, SE of Fairbanks	LL229	21.vi.2006	-146.965	64.471	197	*Picea glauca*	L. Lumley, et al. [1]	5	5	f1 (4), f33 (1)
USA: AK: Hwy 2 mi 82 from Fairbanks	LL230	21.vi.2006	-145.950	64.212	299	*Picea glauca*	L. Lumley, et al. [1]	5	5	f1 (3), o1 (1), o2 (1)
USA: AK: Taylor Hwy, Mile 94	LL235	22.vi.2006	-141.355	64.155	981	*Picea glauca*	L. Lumley, et al. [1]	2	2	f1 (1), bβ (1)
USA: AK: Taylor Hwy, Mile 77	LL234	22.vi.2006	-141.747	64.075	556	*Picea glauca*	L. Lumley, et al. [1]	2	2	o17 (1), bβ (1)
USA: AK: Hwy 2 mi 119 from Fairbanks	LL231	21.vi.2006	-145.149	63.876	378	*Picea glauca*	L. Lumley, et al. [1]	5	5	f1 (5)
USA: AK: North of Denali NP	LL226	20.vi.2006	-149.013	63.843	539	*Picea glauca*	L. Lumley, et al. [1]	5	4	f1 (1), f32 (1), o2 (1), o79 (1)
USA: AK: Taylor Hwy, Mount Fairplay	LL233	22.vi.2006	-142.276	63.704	1015	*Picea glauca*	L. Lumley, et al. [1]	2	2	o1 (1), o55 (1)
USA: AK: Hwy 2, Chief Creek Bridge	LL232	21.vi.2006	-144.014	63.631	474	*Picea glauca*	L. Lumley, et al. [1]	4	4	f1 (2), bβ (2)
USA: AK: Tok	LL205	17.vi.2006	-142.963	63.334	562	*Picea glauca*	L. Lumley, et al. [1]	2	2	f1 (2)
USA: AK: Mile 1269	LL204	17.vi.2006	-142.172	63.201	659	*Picea glauca*	L. Lumley, et al. [1]	3	3	f1 (2), o1 (1)
USA: AK: Chulitna	LL224	20.vi.2006	-149.411	63.151	531	*Picea glauca*	L. Lumley, et al. [1]	2	2	o74 (2)
USA: AK: Cole Creek	LL223	20.vi.2006	-149.746	62.892	389	*Picea glauca*	L. Lumley, et al. [1]	2	2	o1 (1), o74 (1)
USA: AK: Sunshine River	LL221	20.vi.2006	-150.191	62.181	69	*Picea glauca*	L. Lumley, et al. [1]	4	4	o1 (2), o2 (1), o74 (1)
USA: AK: Anchorage	LL214	18.vi.2006	-149.606	61.285	142	*Picea glauca*	L. Lumley, et al. [1]	3	3	bβ (3)
USA: AK: Crow's Nest Mine	LL216	18.vi.2006	-149.084	60.996	205	Picea sitchensis, P. lutzii	L. Lumley, et al. [1]	4	4	o1 (2), o2 (1), o5 (1)

Table 4-1, cont.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSRs (n)	MtDNA (n)	MtDNA Haplotypes (n)
USA: AK: Alyeska	LL215	18.vi.2006	-149.103	60.971	49	Picea sitchensis, P. lutzii	L. Lumley, et al. [1]	4	4	o1 (3), b1 (1)
USA: AK: Girdwood	LL217	19.vi.2006	-149.172	60.941	10	Picea sitchensis, P. lutzii	L. Lumley, et al. [1]	7	7	o1 (1), o2 (2), o74 (4)
USA: AK: Seward / Homer Junction	LL218	19.vi.2006	-149.450	60.509	174	Picea sitchensis, P. lutzii	L. Lumley, et al. [1]	5	5	o1 (4), o74 (1)
USA: AK: Dyea	LL189	15.vi.2006	-135.360	59.498	61	*Picea sitchensis*	L. Lumley, et al. [1]	8	8	o1 (8)
USA: AK: Skagway	LL186	12.vi.2006	-135.316	59.455	7	*Picea sitchensis*	L. Lumley, et al. [1]	5	5	o1 (4), o58 (1)
USA: AK: Haines	LL187	12.vi.2006	-135.459	59.280	29	*Picea sitchensis*	L. Lumley, et al. [1]	5	5	o1 (4), o74 (1)
USA: AZ: West Fork	FS30	13.vii.1995	-111.620	35.189	-	UV light	F. Sperling, J. Powell	1	6	oβ (2), oβ5 (1), oβ6 (3)
USA: AZ: Little Spring	FS27	20.vii.1993	-111.228	34.599	-	*Pseudo. menziesii*	F. Sperling	1	5	oβ (4), oβ11 (1)
USA: AZ: Little Spring	FS28	16.vii.1996	-111.228	34.599	-	UV light	F. Sperling, J. Powell	0	1	c1 (1)
USA: AZ: Walnut Canyon	FS29	14.vii.1996	-110.880	33.230	-	UV light	F. Sperling, J. Powell	0	3	oβ6 (1), oβ7 (1), oβ8 (1)
USA: CA: N of Mount Shasta	LL421	23.vii.2007	-122.131	41.570	1796	*C. fumiferana* lure	L. Lumley	1	1	o2 (1)
USA: CA: N of Mount Shasta	LL421	23.vii.2007	-122.131	41.570	1796	*C. pinus* lure	L. Lumley	5	5	o1 (4), o2 (1)
USA: CA: Mt. Shasta	FS23	9.viii.1996	-122.331	41.343	-	UV light	F. Sperling	1	3	o1 (2), o59 (1)
USA: CA: Warner Mountains	LL419	22.vii.2007	-120.253	41.524	2188	*C. pinus* lure	L. Lumley	1	1	o2 (1)
USA: CA: Warner Mountains	LL419	22.vii.2007	-120.253	41.524	2188	UV light	L. Lumley, et al. [2]	3	3	o1 (2), o2 (1)
USA: CA: Warner Mountains	LL419	22.vii.2007	-120.253	41.524	2188	*C. fumiferana* lure	L. Lumley	2	2	o1 (1), o2 (1)
USA: CA: Bucks Lake Summit	LL413	20.vii.2007	-121.110	39.914	1527	*C. pinus* lure	L. Lumley	1	1	b1 (1)
USA: CA: Bucks Lake Summit	LL413	20.vii.2007	-121.110	39.914	1527	*C. fumiferana* lure	L. Lumley	2	2	b1 (2)
USA: CA: W of Bucks Lake	LL414	20.vii.2007	-121.138	39.837	1550	MV light	L. Lumley, et al. [2]	1	1	b1 (1)
USA: CA: Sierraville	FS31	8.vi.1995	-120.366	39.589	-	UV light	J. Powell	1	5	bβ8 (5)
USA: CA: Sagehen Creek	FS32	25.vii.1996	-120.233	39.433	-	UV light	F. Sperling, D. Rubinoff	6	9	o64 (3), o65 (3), bβ (3)
USA: CA: Tahoe NF, Bowman Rd	LL412	19.vii.2007	-120.668	39.391	1561	*C. pinus* lure	L. Lumley	5	5	o90 (3), b1 (2)
USA: CA: Lake Tahoe	FS34	14.viii.1995	-120.096	39.168	-	UV light	J. Powell	1	5	o82 (1), o90 (1), o84 (1), bβ2 (2)
USA: CA: Angwin	FS22	20.v.1995	-122.448	38.578	-	*Pseudo. menziesii*	F. Sperling	4	5	o1 (3), b1 (2)
USA: CA: Upper Chiquito CG	LL404	10.vii.2007	-119.410	37.505	2143	*C. pinus* lure	L. Lumley, A. Roe	4	4	o78 (1), o90 (1), b1 (2)
USA: CA: Upper Chiquito CG	LL404	10.vii.2007	-119.410	37.505	2143	MV light	L. Lumley, et al. [3]	2	2	o85 (1), b1 (1)
USA: CA: Bass Lake	LL403	9.vii.2007	-119.543	37.356	1434	MV light	L. Lumley, et al. [3]	2	2	b1 (2)
USA: CA: Bass Lake	LL403	9.vii.2007	-119.543	37.356	1434	*C. pinus* lure	L. Lumley, A. Roe	2	2	o78 (1), b1 (1)
USA: CA: Tehachapi	FS34	13.vii.1995	-118.440	35.135	-	*Abies* sp.	F. Sperling, J. Powell	1	5	o86 (3), o87 (1), o88 (1)
USA: CA: Mt. Baldy	FS35	17.vii.1995	-117.551	34.262	-	UV light	F. Sperling, J. Powell	9	9	o1 (1), o75 (1), o76 (1), o77 (1), o83 (1), b1 (2), b2 (2)
USA: CO: Rocky Mtn National Park	FS24	11.vii.1995	-105.068	40.556	-	UV light	P. Opler	2	3	o68(1), oβ3 (1), b1 (1)
USA: CO: Lake City	FS36	26.vii.1996	-107.310	38.029	-	UV light	P. Opler	1	1	o28 (1)
USA: ID: S of Salmon	LL394	4.vii.2007	-114.013	45.077	1618	*Pseudo. menziesii*	L. Lumley, et al. [4]	5	5	o2 (4), o56 (1)
USA: ID: S of Salmon	LL394	4.vii.2007	-114.013	45.077	1618	*Picea glauca*	L. Lumley, et al. [4]	1	1	o2 (1)
USA: MI: Crawford Co.	FS37	3.vii.1997	-84.611	44.680	-	*Pinus banksiana*	B. Bishop	1	5	p1 (5)
USA: MS: Brooklyn	LL724	19.iv.-3.v.1997	-89.186	31.056	-	UV light	R. Kergosien	0	1	bβ (1)
USA: MT: Big Bent Mountains	LL391	4.vii.2007	-111.177	46.333	1544	*Pseudo. menziesii*	L. Lumley, et al. [4]	5	5	o1 (1), o2 (2), o28 (1), o45 (1)
USA: MT: E of Continental Divide on Hwy 43	LL393	4.vii.2007	-113.710	45.653	1917	*Pseudo. menziesii*	L. Lumley, et al. [4]	5	5	o2 (2), o44 (1), o81 (1), oβ (1)
USA: MT: E of Continental Divide on Hwy 43	LL393	4.vii.2007	-113.710	45.653	1917	*Juniperus sp.*	L. Lumley, et al. [4]	1	1	o2 (1)
USA: MT: E of Continental Divide on Hwy 43	LL393	4.vii.2007	-113.710	45.653	1917	*Picea glauca*	L. Lumley, et al. [4]	1	1	o2 (1)
USA: MT: E of Wise River	LL392	4.vii.2007	-112.901	45.781	1336	*Pseudo. menziesii*	L. Lumley, et al. [4]	1	1	o2 (1)
USA: MT: Little Bent Mountains 1	LL388	3.vii.2007	-110.912	46.804	1706	*Juniperus sp.*	L. Lumley, et al. [4]	1	1	o17 (1)
USA: MT: Little Bent Mountains 1	LL388	3.vii.2007	-110.912	46.804	1706	*Pseudo. menziesii*	L. Lumley, et al. [4]	4	4	o2 (3), o39 (1)
USA: MT: Little Bent Mountains 2	LL389	3.vii.2007	-110.893	46.820	1757	*Picea glauca*	L. Lumley, et al. [4]	2	2	o1 (1), o2 (1)
USA: MT: Montana City	LL719	27.vii.2005	-111.930	46.540	-	UV light	T. Simonsen	1	1	oβ (1)
USA: NM: NE of Hyde State Park	FS38	8.vii.2001	-105.892	35.714	-	UV light	J. Powell	2	2	oβ (1), oβ4 (1)
USA: NV: Mt. Charleston	FS39	22.vii.1996	-116.250	36.417	-	UV light	F. Sperling, J. Powell	10	10	o66 (4), o67 (1), oβ9 (4), oβ10 (1)

Table 4-1, cont.

Sample Locality	ID	Coll. Date	Long. (deg)	Lat. (deg)	Elev. (m)	Larval Host / Coll. Method	Collectors	SSRs (n)	MtDNA (n)	MtDNA Haplotypes (n)
USA: NV: Timber Creek	FS40	18.vii.1996	-114.878	39.249	-	UV light	F. Sperling, J. Powell	1	5	o2 (3), b1 (1), oβ2 (1)
USA: NY: Ithaca	LL720	3.vii.1975	-76.503	42.444	335	UV light	J. Franclemont	0	1	bβ (1)
USA: OR: E of Santiam Pass	LL424	25.vii.2007	-121.707	44.426	987	*C. fumiferana* lure	L. Lumley	2	2	o2 (1), o28 (1)
USA: OR: Jack Creek	LL425	25.vii.2007	-121.694	44.489	906	UV light	L. Lumley, et al. [2]	4	4	o1 (1), o2 (2), o39 (1)
USA: OR: Lemolo Lake	LL422	24.vii.2007	-122.112	43.312	1294	UV light	L. Lumley, et al. [2]	1	1	o1 (1)
USA: OR: Lemolo Lake	LL422	24.vii.2007	-122.112	43.312	1294	*C. fumiferana* lure	L. Lumley	3	3	o1 (1), o28 (2)
USA: OR: Mount Hood NF 1	LL426	26.vii.2007	-121.551	45.188	1069	*C. fumiferana* lure	L. Lumley	5	5	o28 (4), o31 (1)
USA: OR: Mount Hood NF 2	LL427	26.vii.2007	-121.571	45.192	1146	*C. pinus* lure	L. Lumley	1	1	o28 (1)
USA: OR: Mount Hood NF 2	LL427	26.vii.2007	-121.571	45.192	1146	*C. fumiferana* lure	L. Lumley	5	5	o1 (1), o2 (4)
USA: OR: Mount Hood NF 3	LL428	26.vii.2007	-121.603	45.213	906	*C. fumiferana* lure	L. Lumley	3	3	o1 (3)
USA: OR: Mount Hood NF 4	LL429	26.vii.2007	-121.615	45.213	922	*C. fumiferana* lure	L. Lumley	1	1	o2 (1)
USA: OR: Mount Hood NF 5	LL430	26.vii.2007	-121.627	45.232	947	UV light	L. Lumley, et al. [2]	3	3	o2 (2), o71 (1)
USA: OR: W of Santiam Pass	LL423	25.vii.2007	-121.957	44.439	1369	*C. fumiferana* lure	L. Lumley	5	5	o2 (2), o28 (3)
USA: TN: Great Smoky Mountains, Cosby	LL723	9.vi.2002	-83.215	35.776	-	UV light	R. Brown, S. Lee	1	0	
USA: TN: Great Smoky Mountains, Foothills	LL722	9.vi.2002	-83.220	35.816	-	UV light	R. Brown, S. Lee	1	1	bβ10 (1)
USA: UT: Clear Creek	LL398	6.vii.2007	-113.320	41.953	1918	*C. fumiferana* lure	L. Lumley, A. Roe	7	7	o1 (1), o2 (3), o11 (1), o28 (1), o31 (1)
USA: UT: Clear Creek	LL398	6.vii.2007	-113.320	41.953	1918	MV light	L. Lumley, et al. [3]	2	2	o2 (2)
USA: UT: DuckCreek	FS41	21.vii.1996	-111.099	41.789	-	UV light	F. Sperling, J. Powell	7	10	o1 (1), o66 (1), bβ3 (1), bβ4 (3), oβ (4)
USA: UT: E of Logan	LL397	5.vii.2007	-111.630	41.780	1709	*C. fumiferana* lure	L. Lumley, A. Roe	3	3	o28 (3)
USA: UT: E of Logan	LL397	5.vii.2007	-111.630	41.780	1709	MV light	L. Lumley, et al. [3]	2	2	o2 (1), o28 (1)
USA: UT: Ephraim Canyon	FS42	19.vii.1996	-111.584	39.360	-	UV light	F. Sperling, J. Powell	9	10	o2 (1), o11 (1), o28 (1), o38 (1), o57 (1), bβ5 (5)
USA: WA: Chiwawa River	LL433	28.vii.2007	-120.785	47.965	1059	*C. fumiferana* lure	L. Lumley	3	3	o1 (1), o2 (1), o5 (1)
USA: WA: Chiwawa River	LL433	28.vii.2007	-120.785	47.965	1059	UV light	L. Lumley, et al. [2]	3	3	o1 (2), o28 (1)
USA: WA: Chiwawa River	LL433	28.vii.2007	-120.785	47.965	1059	*C. pinus* lure	L. Lumley	2	2	o1 (2)
USA: WA: E of Usk	LL434	29.vii.2007	-117.186	48.394	1100	*C. fumiferana* lure	L. Lumley	3	3	o1 (1), o2 (2)
USA: WA: E of Usk	LL434	29.vii.2007	-117.186	48.394	1100	UV light	L. Lumley, et al. [2]	2	2	o2 (1), o25 (1)
USA: WA: E of Usk	LL434	29.vii.2007	-117.186	48.394	1100	*C. pinus* lure	L. Lumley	4	4	o1 (1), o2 (1), o28 (1), o44 (1)
USA: WA: Trout Creek Road	LL432	27.vii.2007	-121.715	46.080	1054	UV light	L. Lumley, D. Lawrie	3	3	o2 (1), o28 (1), o30 (1)
USA: WA: Trout Creek Road	LL432	27.vii.2007	-121.715	46.080	1054	*C. fumiferana* lure	L. Lumley	3	3	o1 (1), o2 (1), o28 (1)
FRA: Alsace	FS20	31.v.1996	-7.333	47.750	-	UV light	M. Kenis	0	1	m1 (1)

Collectors, abbreviations:
L. Lumley, et al. [1]: L. Lumley, E. Lumley, M. Lumley
L. Lumley, et al. [2]: L. Lumley, J. Dombroskie, D. Lawrie, A. Rose
L. Lumley, et al. [3]: L. Lumley, M. Djernais, J. Dombroskie, D. Lawrie, A. Roe, A. Rose, T. Simonsen
L. Lumley, et al. [4]: L. Lumley, D. Lawrie, A. Roe
FIDS: Canadian Forest Insect and Disease Survey
ASRD: Alberta Sustainable Resource Development
MCFB: Manitoba Conservation, Forestry Branch
NBNR: New Brunswick Natural Resources
NSDNR: Nova Scotia Department of Natural Resources
OMNR: Ontario Ministry of Natural Resources
QMNRW: Quebec Ministry of Natural Resources and Wildlife
USFS: United States Forest Service

Table 4-2: Putative adaptive traits used to identify *Choristoneura* to species, based on descriptions previously used for delimitation and identification along with current observations (Freeman, 1967; Harvey, 1985; Powell, 1995; Lumley & Sperling, 2010).

Life History Traits:

		Bioregion	Larval diapause	Host plant association	Pheromone attraction
Species	*C. fumiferana*	Boreal	1 year cycle	white spruce, balsam fir	aldehyde blend
	C. pinus	Boreal	1 year cycle	jack pine, eastern white pine	acetate blend
	C. occidentalis	Cordilleran	1 year cycle	Douglas-fir, occasionally spruce	aldehyde blend
	C. biennis	Cordilleran	2 year cycle	Engelmann spruce, subalpine fir	aldehyde blend
	C. orae	Coastal	1 or 2 year cycle	Sitka spruce, lutz (Sitka x white) spruce	acetate blend
	C. orae 'Inland'	Cordilleran - northern	1 or 2 year cycle	white spruce, lutz (Sitka x white) spruce	unknown
	C. lambertiana	Cordilleran	1 year cycle	pine species	acetate blend
	C. lambertiana 'North'	Cordilleran	unknown	lodgepole pine, but mainly unknown	acetate blend
	C. retiniana	Cordilleran - SW USA	1 year cycle	True fir *(Abies)* spp.	acetate blend
	C. carnana	Cordilleran - SW USA	1 year cycle	Douglas-fir, bigcone Douglas-fir	aldehyde blend

Morphology:

		Forewing colour & pattern
Species	*C. fumiferana*	gray except some females are reddish-brown with black scales
	C. pinus	reddish-brown with distinct bands, darker transverse striations, no black scales; noticeably small
	C. occidentalis	reddish-brown, usually with black scales
	C. biennis	gray, dark gray or dark reddish-brown; with black scales; usually noticeably large
	C. orae	females reddish-brown, males grey
	C. orae 'Inland'	similar to northern species (*C. fumiferana* , *C. biennis* , *C. orae*)
	C. lambertiana	reddish-brown to reddish-tan, bands suffused (run together), no black scales; usually noticeably small
	C. lambertiana 'North'	reddish-brown, often with black longitudinal band
	C. retiniana	pale goldish-tan or tawny, with or without banding pattern
	C. carnana	reddish-brown, sub-median band distinct but without lines, no to few lines throughout wing, usually without black scales

Table 4-3. MtDNA haplotypes with corresponding GenBank accession numbers and references.

Haplotype	Accession No.	References
f1	L19098[a]	Lumley & Sperling, 2010; Roe & Sperling, 2007; Sperling & Hickey, 1994
f2	GQ890278[a]	Lumley & Sperling, 2010; Roe & Sperling, 2007; Sperling & Hickey, 1994
f3	GQ890279	Lumley & Sperling, 2010; Sperling & Hickey, 1994
f4	HM223075	Previously unpublished
f5	HM223076	Previously unpublished
f7	HM223078	Previously unpublished
f9	GQ890280	Lumley & Sperling, 2010
f10	GQ890281	Lumley & Sperling, 2010
f11	GQ890282	Lumley & Sperling, 2010
f13	HM223081	Previously unpublished
f15	HM223083	Previously unpublished
f17	GQ890283	Lumley & Sperling, 2010
f25	HM223092	Previously unpublished
f26	HM223093	Previously unpublished
f27	HM223094	Previously unpublished
f28	HM223095	Previously unpublished
f29	HM223096	Previously unpublished
f30	HM223097	Previously unpublished
f31	HM223098	Previously unpublished
f32	HM223099	Previously unpublished
f33	HM223100	Previously unpublished
f34	HM223101	Previously unpublished
p1	L19095[a]	Lumley & Sperling, 2010; Roe & Sperling, 2007; Sperling & Hickey, 1994
p2	HM223102	Previously unpublished
p3	GQ890284	Lumley & Sperling, 2010
p4	GQ890285	Lumley & Sperling, 2010
p5	GQ890286	Lumley & Sperling, 2010
p6	GQ890287	Lumley & Sperling, 2010
p7	GQ890288	Lumley & Sperling, 2010
p8	GQ890289	Lumley & Sperling, 2010
p9	HM223103	Previously unpublished
p10	HM223104	Previously unpublished
p11	HM223105	Previously unpublished
p12	HM223106	Previously unpublished
p13	HM223107	Previously unpublished
o1	L19094, DQ792584[a]	Lumley & Sperling, 2010; Roe & Sperling, 2007; Sperling & Hickey, 1994
o2	GQ890290	Lumley & Sperling, 2010; Sperling & Hickey, 1994
o3	HM223108	Previously unpublished
o4	HM223109	Previously unpublished
o5	GQ890291	Lumley & Sperling, 2010; Sperling & Hickey, 1994
o6	HM223110	Previously unpublished
o7	HM223111	Previously unpublished
o8	HM223112	Previously unpublished
o9	HM223113	Previously unpublished
o11	GQ890292	Lumley & Sperling, 2010
o13	HM223116	Previously unpublished
o14	HM223117	Previously unpublished
o17	HM223120	Previously unpublished
o18	HM223121	Previously unpublished
o20	HM223123	Previously unpublished
o24	HM223127	Previously unpublished
o25	HM223128	Previously unpublished
o28	HM223131	Previously unpublished

Table 4-3, cont.

Haplotype	Accession No.	References
o30	HM223133	Previously unpublished
o31	HM223134	Previously unpublished
o32	HM223135	Previously unpublished
o33	HM223136	Previously unpublished
o34	HM223137	Previously unpublished
o35	HM223138	Previously unpublished
o36	HM223139	Previously unpublished
o37	HM223140	Previously unpublished
o38	HM223141	Previously unpublished
o39	HM223142	Previously unpublished
o40	HM223143	Previously unpublished
o41	HM223144	Previously unpublished
o42	HM223145	Previously unpublished
o43	HM223146	Previously unpublished
o44	HM223147	Previously unpublished
o45	HM223148	Previously unpublished
o46	HM223149	Previously unpublished
o47	HM223150	Previously unpublished
o48	HM223151	Previously unpublished
o49	HM223152	Previously unpublished
o50	HM223153	Previously unpublished
o51	HM223154	Previously unpublished
o52	HM223155	Previously unpublished
o53	HM223156	Previously unpublished
o54	HM223157	Previously unpublished
o55	HM223158	Previously unpublished
o56	HM223159	Previously unpublished
o57	HM223160	Previously unpublished
o58	HM223161	Previously unpublished
o59	HM223162	Previously unpublished
o60	HM223163	Previously unpublished
o61	HM223164	Previously unpublished
o62	HM223165	Previously unpublished
o63	HM223166	Previously unpublished
o64	HM223167	Previously unpublished
o65	HM223168	Previously unpublished
o66	HM223169	Previously unpublished
o67	HM223170	Previously unpublished
o68	HM223171	Previously unpublished
o69	HM223172	Previously unpublished
o70	HM223173	Previously unpublished
o71	HM223174	Previously unpublished
o72	HM223175	Previously unpublished
o73	HM223176	Previously unpublished
o74	HM223177	Previously unpublished
o75	HM223178	Previously unpublished
o76	HM223179	Previously unpublished
o77	HM223180	Previously unpublished
o78	HM223181	Previously unpublished
o79	HM223182	Previously unpublished
o80	HM223183	Previously unpublished
o81	HM223184	Previously unpublished
o82	HM223185	Previously unpublished
o83	HM223186	Previously unpublished

Table 4-3, cont.

Haplotype	Accession No.	References
o84	HM223187	Previously unpublished
o85	HM223188	Previously unpublished
o86	HM223189	Previously unpublished
o87	HM223190	Previously unpublished
o88	HM223191	Previously unpublished
o89	HM223192	Previously unpublished
o90	HM223193	Previously unpublished
o91	HM223194	Previously unpublished
o92	HM223195	Previously unpublished
o93	HM223196	Previously unpublished
b1	DQ792586, DQ792587[a]	Lumley & Sperling, 2010; Roe & Sperling, 2007; Sperling & Hickey, 1994
b2	HM223197	Previously unpublished
b3	HM223198	Previously unpublished
oB	DQ792585[a]	Roe & Sperling, 2007; Sperling & Hickey, 1994
oB2	HM223199	Previously unpublished
oB3	HM223200	Previously unpublished
oB4	HM223201	Previously unpublished
oB5	HM223202	Previously unpublished
oB6	HM223203	Previously unpublished
oB7	HM223204	Previously unpublished
oB8	HM223205	Previously unpublished
oB9	HM223206	Previously unpublished
oB10	HM223217[a]	Previously unpublished
oB11	HM223207	Previously unpublished
oB12	HM223208	Previously unpublished
bB	L19096[a], L19097	Roe & Sperling, 2007; Sperling & Hickey, 1994
bB2	HM223209	Previously unpublished
bB3	HM223210	Previously unpublished
bB4	HM223211	Previously unpublished
bB5	HM223212	Previously unpublished
bB6	HM223213	Previously unpublished
bB7	HM223214	Previously unpublished
bB8	HM223218[a]	Previously unpublished
bB9	HM223215	Previously unpublished
bB10	HM223216	Previously unpublished
c1	GQ890293[a]	Lumley & Sperling, 2010
m1	GQ890294[a]	Lumley & Sperling, 2010
r1	L19099[a]	Sperling & Hickey, 1994
r2	GQ890295	Lumley & Sperling, 2010; Sperling & Hickey, 1994

[a] Sequence analyzed for 2.3 kb COI and COII region

Table 4-4: Total number of *Choristoneura* individuals within each species, identified by adaptive traits as per Table 4-2, with numbers of each mitochondrial DNA lineage and simple sequence repeat assignment (population).

		mtDNA lineage					mtDNA	SSR assignment						SSR
		f	**p**	**o**	**oβ**	**bβ**	**total**	**1**	**2**	**3**	**4**	**5**	**6**	**total**
Species	***C. fumiferana***	252	0	7	0	0	259	270	0	2	2	0	0	274
	C. pinus	0	163	2	0	4	169	3	0	0	0	142	0	145
	C. occidentalis	0	0	213	29	0	242	0	29	223	3	0	1	256
	C. biennis	0	0	203	3	3	209	2	46	153	0	0	0	201
	C. orae	0	0	57	0	3	60	0	49	8	0	0	0	57
	C. orae 'Inland'	0	0	33	0	13	46	1	21	25	0	0	0	47
	C. lambertiana	0	0	14	1	7	22	0	1	0	14	0	0	15
	C. lambertiana 'North'	0	0	75	0	13	88	1	15	66	6	0	0	88
	C. retiniana	0	0	38	5	12	55	0	1	5	0	0	34	40
	C. carnana	0	0	12	1	0	13	0	1	11	0	0	0	12
	Total	252	163	654	39	55	1163	277	163	493	25	142	35	1135

Table 4-5. Number of *Choristoneura* individuals containing each of the possible 30 genetic combinations of mtDNA lineage and SSR assignment (k=6).

		mtDNA Lineage				
		f	**p**	**o**	**oβ**	**bβ**
SSR Assignment	**Pop 1** - *fumiferana*	231	3	8	0	0
	Pop 2 - Western A	0	0	144	3	9
	Pop3 - Western B	2	0	428	16	21
	Pop 4 - *lambertiana*	2	0	16	2	4
	Pop5 - *pinus*	0	136	1	0	2
	Pop6 - *retiniana*	0	0	24	5	6

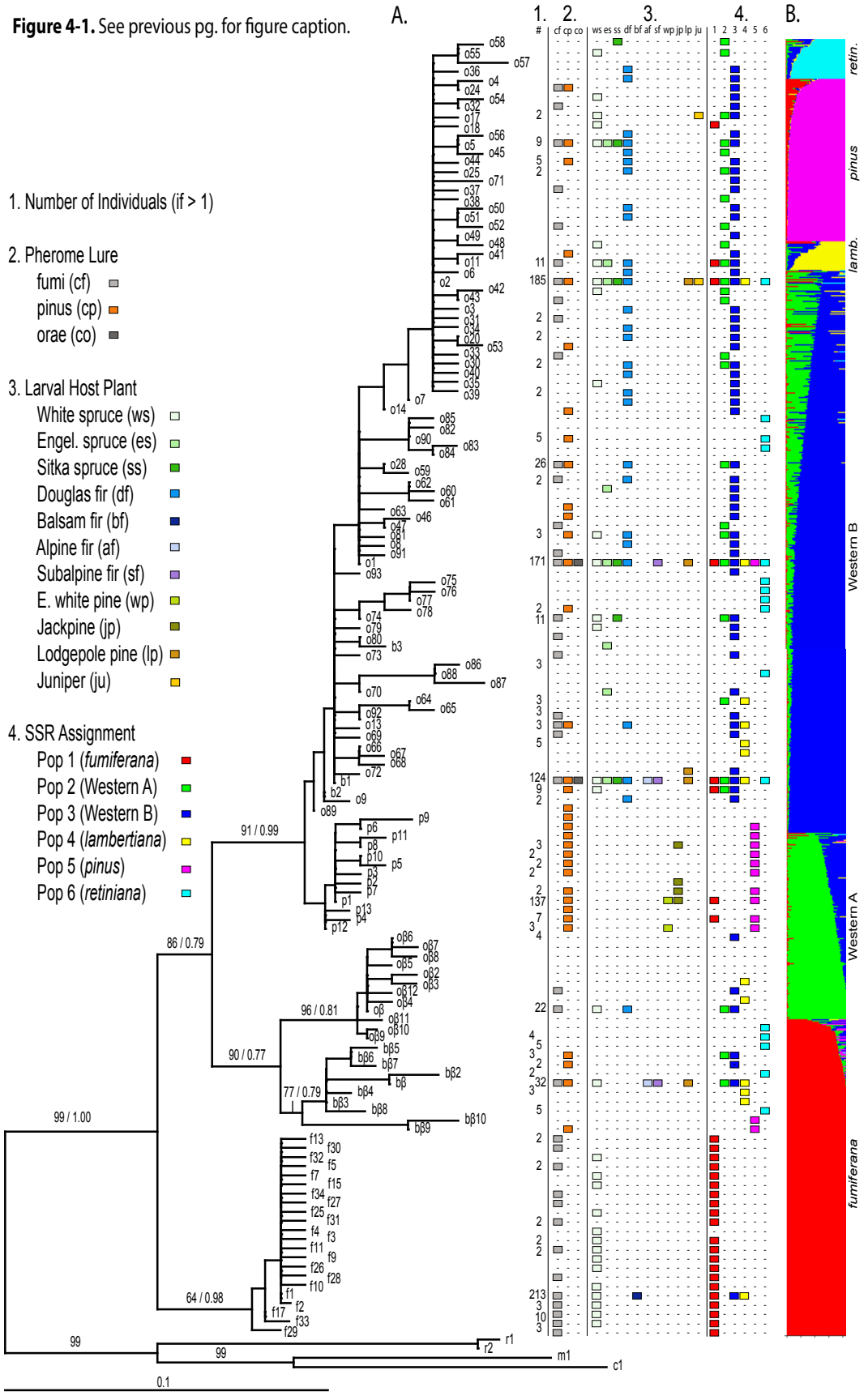
Pop = population

Western A and B include *C. occidentalis* , *C. biennis* , *C. orae* , *C. orae ' Inland'* , *C. lambertiana* , *C. lambertiana 'North'* , *C. retiniana* , and *C. carnana* .

Following Page:

Figure 4-1. A. Maximum likelihood tree for 142 unique ingroup haplotypes found in the *Choristoneura fumiferana* species complex. The analysis included a 2.3 kb region of COI and COII mitochondrial DNA for 12 individuals and a 470 bp region of COI mitochondrial DNA for 1155 individuals. Maximum likelihood bootstrap values and Bayesian support values are indicated for the main lineages. Beside each haplotype is phenotype information for specimens containing that haplotype, including: 1) number of specimens, 2) pheromone attraction, 3) larval host plant, and 4) assigned SSR population at k=6. **B.** Bar plot showing the probability of simple sequence repeat assignment using Structure analysis (k=6) for 1135 individuals, grouped by population.

Figure 4-1. See previous pg. for figure caption.



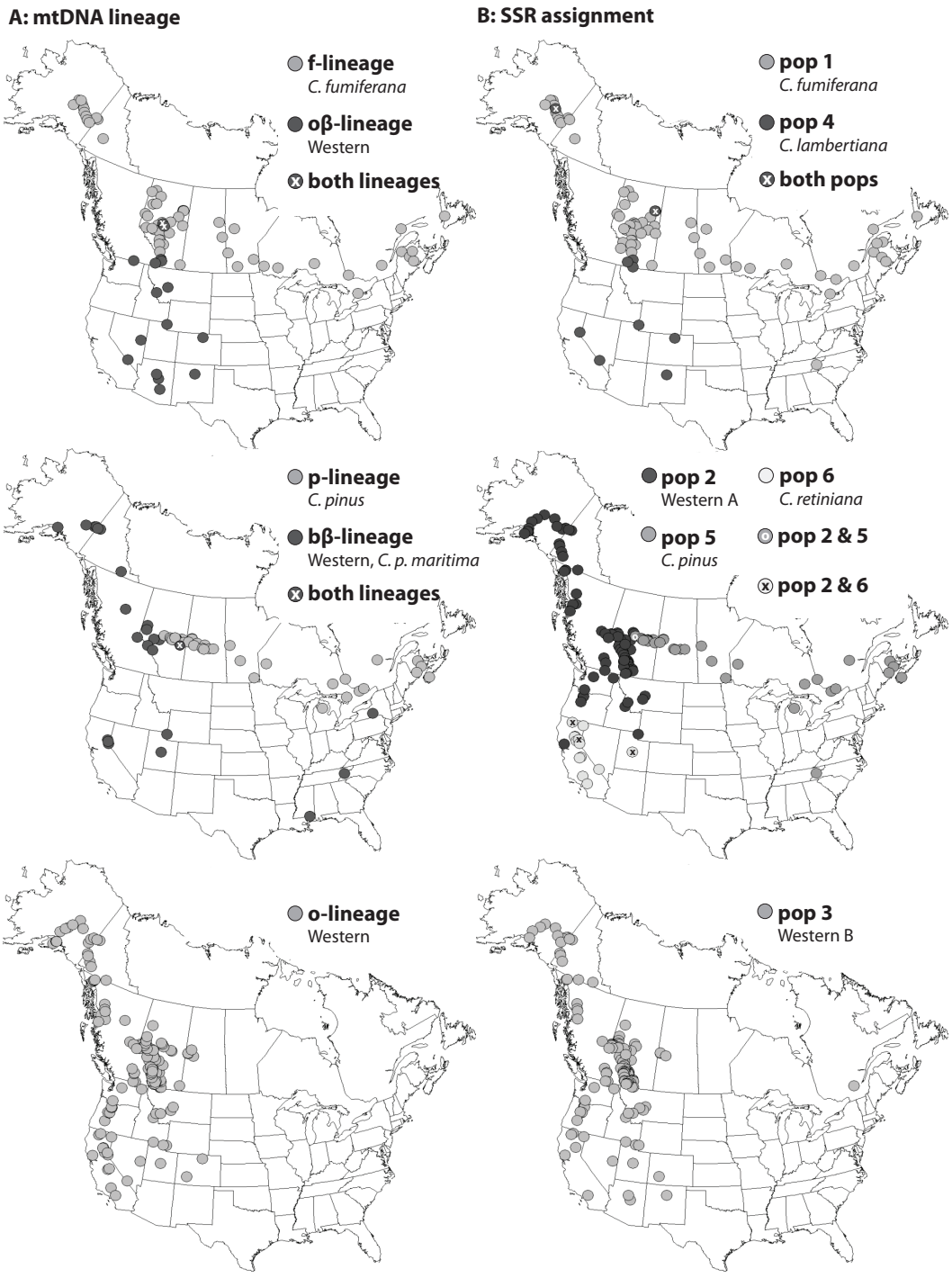


Figure 4-2. Geographical distributions of genetic variation in the *C. fumiferana* species complex for: A. Five major mtDNA lineages; and B. SSR assignments at k=6 using Structure (Pritchard *et al.*, 2000). MtDNA lineages and SSR assignments are identified by species names where they primarily associate with one species phenotype, as identified by the description given in Table 4-2.

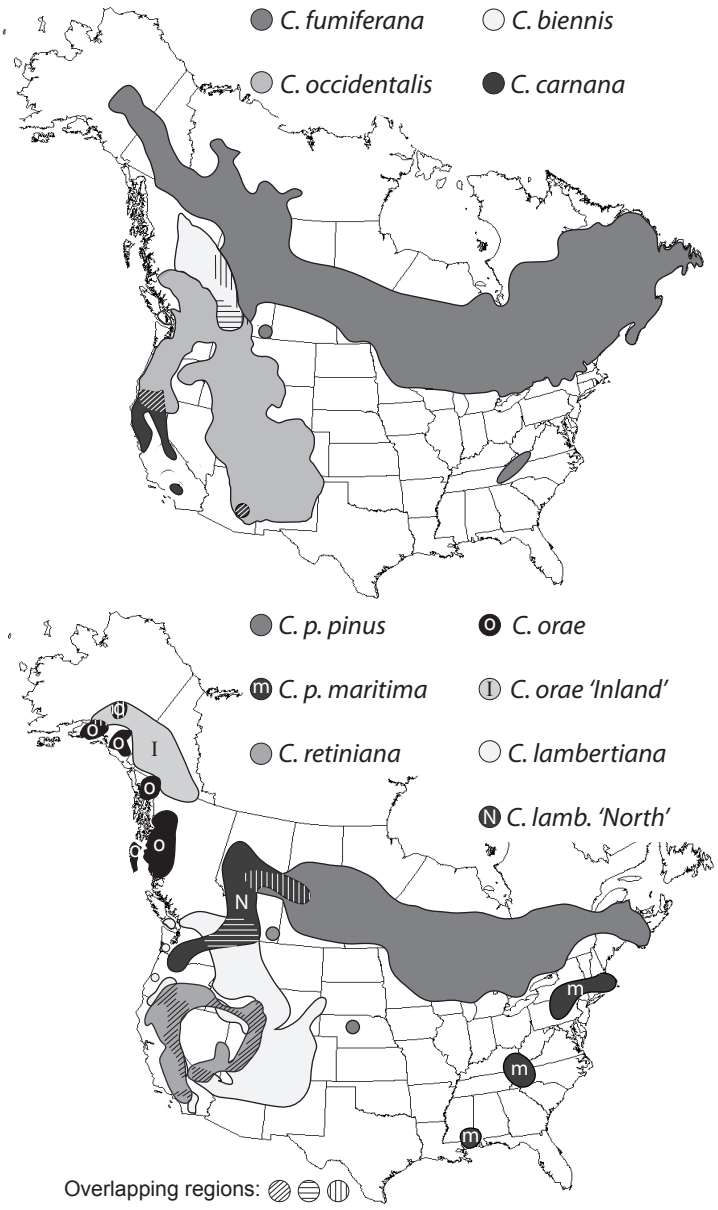


Figure 4-3. Distributions of coniferophagous *Choristoneura* species, amended from Harvey (1985) to include findings from this study.

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Chapter 5

Identification of sympatric species from the spruce budworm (*Choristoneura fumiferana*) species complex on a forest island

Introduction

Islands have long fascinated biogeographers, ecologists and evolutionary biologists, providing insights into the mechanisms that shape biological diversity (MacArthur & Wilson, 1967; Brown & Lomolino, 2000; Gillespie & Roderick, 2002; Lomolino & Brown, 2009). Island biogeography theory encompasses the biota of oceanic islands, isolated continental habitats such as lakes or mountain tops (Hughes & Eastwood, 2006; Thornton, 2007) and habitats that have been fragmented through human activity (Harris, 1984; Haila, 2002; Thornton, 2007). However, this theory focuses on the number of species on an island as an equilibrium between colonization and extinction of species, implying little change in the species themselves, and is largely mute on the subject of species identification and delimitation on islands. Nonetheless, processes of divergence, hybridization, or speciation may occur on an island, particularly since adaptive traits may be under increased selection pressure to adapt to a new environment (e.g. Grant *et al.*, 1996; Hughes & Eastwood, 2006). An increased propensity to hybridize and exchange genes may also be exhibited between closely related species in new contact on an island (e.g. Clarke *et al.*, 1996; Grant *et al.*, 1996). My study focused on a continental island, Cypress Hills, where these challenges have complicated the identification of conifer feeding species present in the spruce budworm (*Choristoneura fumiferana*) complex (Lepidoptera: Tortricidae).

Cypress Hills is located on the border of southern Alberta and Saskatchewan in Canada, and is a forested continental island isolated from the nearest coniferous region by approximately 250 km (Chilton, 2003). Coniferous tree species include pine (*Pinus contorta* Douglas ex Louden) and spruce [*Picea*

glauca (Moench) Voss and *Picea albertiana* S. Brown emend. Strong & Hills]. *Picea albertiana* are hybrid forms of *P. glauca* (Moench) Voss and *P. engelmannii* Parry ex Engelmann (Strong & Hills, 2006). Based on pollen records, Cypress Hills was a refugium that, due to its higher elevation and higher rainfall, allowed *Picea* and *Pinus* species to survive while surrounding regions developed into grassland somewhere between 12,000 to 14,000 years before present (Strong & Hills, 2005).

The presence of these coniferous trees has allowed the colonization of insect species from the spruce budworm (*Choristoneura fumiferana* Clemens) species group, a coniferophagous pest complex that ranges across the Nearctic region. This is an extremely well studied species group of moths that includes *C. fumiferana*, which is the most destructive insect defoliator in North America (Volney & Fleming, 2007) and has become a model organism for studying insect outbreak dynamics (e.g. Greenbank *et al.*, 1980; Williams & Liebhold, 2000; Royama *et al.*, 2005; Régnière & Nealis, 2007). Species within the complex are not all of equal economic importance as some are less likely to go into an outbreak phase and cause widespread damage. The species also differ in their morphology, behaviour, and bioregion association. However, these differences are frequency-related rather than set for each species (Harvey, 1985; Dang, 1992; Harvey, 1997), and so multiple characters are typically necessary for species identification. Although adaptive traits work well for identification when spruce budworm species are found in their typical habitats, identifying species outside of their known range and in locations with non-typical environmental characteristics, such as Cypress Hills, can be very difficult. Reputedly neutral genetic markers like mitochondrial DNA (mtDNA) and simple sequence repeats (SSRs, also referred to as microsatellites) can help to identify some species, but adaptive traits such as life-history traits and morphology are still necessary to identify most of the species, requiring an integrative approach using both neutral and adaptive markers (Chapter 4). Sperling and Hickey (1994) identified two mtDNA lineages from the *Choristoneura fumiferana* species complex in Cypress Hills. These were the f-lineage which is associated with *C. fumiferana*, and the o-lineage which is

associated with all species residing in western North America (Sperling & Hickey, 1994; Lumley & Sperling, 2010; Chapter 4). Since species share mtDNA lineages, more work including studying adaptive traits is necessary to determine which species from the *C. fumiferana* complex are in residence in Cypress Hills.

The Cypress Hills habitat island is located between two major ecosystem regions hosting different species of the spruce budworm species complex. About 400 km to the north and east is the boreal region with *C. fumiferana* Clemens and *C. pinus* Freeman. To the west is a mountainous region with the remaining six species within the complex: *C. occidentalis* Freeman, *C. biennis* Freeman, *C. orae* Freeman, *C. retiniana* Walsingham, *C. carana* Barnes and Busck, and *C. lambertiana* Busck. Described species that range geographically closest to Cypress Hills in the cordilleran region 250 km to the west include *C. occidentalis*, *C. biennis*, and *C. lambertiana*. A host plant connection between the boreal and cordilleran regions has been hypothesized by Strong & Hills (2005), potentially allowing refugial populations of several spruce budworm species to continue to exist for up to 14,000 years before present. These species are also known to migrate long distances, having been observed to disperse as far as 600 km (Dobesberger *et al.*, 1983), which would allow more recent dispersal events from either the boreal or cordilleran regions to populate Cypress Hills.

The hybrid origin of one of the spruce species in Cypress Hills, *Picea albertiana*, points towards the possibility that resident spruce budworm populations in Cypress Hills may also be hybrids, formed through secondary contact of 'mainland' boreal and cordilleran species; all species within the spruce budworm complex have been shown to hybridize and produce viable offspring in laboratory studies (Harvey, 1997). With Cypress Hills containing only three conifer species there is also the possibility of increased rates of selection, leading to speciation, for species that have immigrated from other regions but do not originally prefer spruce (*Picea albertiana* or *Picea glauca*) or lodgepole pine. Colonizing or refugial species may also have to adapt to different environmental conditions in Cypress Hills compared to typical conditions in their normal range, again possibly leading to increased rates of selection and speciation.

With these considerations in mind, my objectives were to determine the identity of the spruce budworm species residing in Cypress Hills, Canada and to determine what characteristics may allow them to maintain their genomic integrity if there are multiple species. Putatively neutral markers (SSRs and mtDNA) were analysed to measure gene flow and to determine whether there were any hybridization events among species or populations within Cypress Hills, as well as to assign individuals to previously delimited species by comparing them to 'mainland' species. Adaptive traits (larval host plant, pheromone attraction, adult flight phenology, and adult forewing morphometrics) were surveyed to determine if there were any evolutionarily significant characters that may allow species to maintain their genomic integrity while existing in sympatry, as well as to assist in assigning Cypress Hills individuals to species by comparing their traits with those found in mainland species. My work is intended to contribute to our understanding of spruce budworm species interactions as well as to explore appropriate methods for delimiting and identifying species on islands.

Materials & Methods

Collections for Cypress Hills

As indicated in Figure 5-1 and Table 5-1, collected specimens that were used in this study came from 12 locations in Cypress Hills, and included both larval and pheromone collections which are further described below.

Larval Collections

In 2005, larvae feeding on spruce were sampled from four locations in Cypress Hills (Table 5-1, Figure 5-1), with two of the localities (Firerock and Battle Creek) having higher population numbers and providing more extensive collections. A few larval samples were also taken from spruce in 2008. Extensive searches were conducted across the region in 2006 and 2008 for larvae feeding on lodgepole pine, resulting in three individuals collected in 2008 from three localities. Larvae were fed host foliage and reared to the adult stage. Larval head

capsule colouration, larval diapause characteristics, and date of adult emergence were recorded along with typical locality information.

Adult Collections

During July 8-11 in 2006, adults were collected using pheromone traps from across Cypress Hills in Alberta and Saskatchewan, of which 7 locations were selected for further processing as listed in Table 5-1. Trap localities included locations containing mainly spruce or lodgepole pine as well as mixed stands. Two green unitraps (Contech, Victoria, BC) were set out 75 m apart and at a height of 2.75 m within each locality, with one trap containing *C. fumiferana* lure and one trap containing *C. pinus pinus* lure. The *C. fumiferana* pheromone lures consisted of 95:5 (*E*,*Z*)- 11-tetradecenal (Contech). The *C. p. pinus* pheromone lures contained a 9:1 ratio of 85:15 (*E*,*Z*)-11-tetradecenyl acetates and 85:15 (*E*,*Z*)-11-tetradecen-1-ols (Silk *et al.*, 1985) and were from the Canadian Forest Service. Vapona (Contech) was used as a killing agent. Blacklight traps were also placed out in these same locations for one trap night per locality over the three night collecting period, with ethyl acetate used as a killing agent. Longitude, latitude, elevation, and coniferous tree species were recorded for each location. Adults were transported back to the lab and frozen at -20 ^{o}C to await further processing.

In 2008, pheromone traps were placed in nine localities across the region (Table 5-1, Figure 5-1) with localities mainly being chosen on the basis of host plant stand. These included the same localities sampled for larvae in 2005 (Firerock, Spruce Coulee, Reesor Lake, Battle Creek), and many of the same localities sampled for adults in 2006 (Firerock, Willow Creek, east of Spruce Coulee Road, Spruce Coulee, Reesor Lake, Grayburn). Three localities mainly contained spruce, three mainly contained lodgepole pine and three localities were mixed stands, although all stands had some portion of either species. Three unitraps were set out for each locality, one trap containing *C. fumiferana* lure, one trap containing *C. pinus pinus* lure, and one control trap containing no lure. The pheromone traps were placed in the field on June 27, 2008, and trap catches were

collected and counted every ten days until September 5, 2008, for a total of seven trap collections. Recorded location information included longitude, latitude, elevation, and coniferous tree species. Adults were placed at -20 ^{o}C to await further processing. Voucher specimens and images were deposited at the University of Alberta in the E. H. Strickland Entomological Museum.

Collections Outside of Cypress Hills

Locality and collection information for spruce budworm species collected outside of Cypress Hills are compiled in Chapter 4. In addition, collection information for species that acted as outgroups for mtDNA analysis (*C. rosaceana* Harris, *C. conflictana* Walker, and *C. murinana* Hübner) is summarized in Chapter 4.

Mitochondrial DNA

A 470 bp region of mtDNA was amplified and sequenced from the COI gene using the methods recorded in Lumley and Sperling (2010). This region was chosen as it contains higher sequence divergence compared to other regions within the COI and COII regions of mtDNA for the spruce budworm species complex, and therefore is the most informative and economical region to sequence and analyze (Sperling & Hickey, 1994; Roe & Sperling, 2007a ; Lumley & Sperling, 2010). A total of 112 larval samples from 2005, 69 adult trap catch samples from 2006, and 296 larval and pheromone trap catch samples from 2008 were sequenced successfully for a total of 477 moths. From the 2006 collection, all specimens collected using the *C. pinus* lure were sequenced (n=9), plus three locations were chosen from which 10 moths were sequenced from each of the *C. fumiferana* pheromone trap and blacklight light trap catches. For the 2008 pheromone trap catches, at least three specimens were sequenced from every trap at every sampling date (including the control trap), unless fewer were collected in which case the one or two specimens available were sequenced.

MtDNA sequence was assembled and checked for ambiguities in Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI), then aligned by eye in

PAUP 4.0b10 (Swofford, 2003). All haplotypes previously recorded from across North America from 1167 specimens (Chapter 4) were added to the analysis, including those sequenced for the same 470 bp region as well as 12 haplotypes sequenced for the full 2.3 kb region of COI and COII. Four outgroup specimens (2 x *C. rosaceana* Harris, *C. conflictana* Walker, and *C. murinana* Hübner) were used. Sequence for the larger 2.3 kb region of mtDNA was included to maximize the phylogenetic informativeness and basal stability of the tree. Individual sequences from the Cypress Hills specimens were then reduced to unique haplotypes using MacClade v4.08 (Maddison & Maddison, 2005). New haplotypes were deposited in GenBank (Table 5-2).

MtDNA sequence was analysed using the same methods as in Chapter 4, using CIPRES portals v1.15 and v2.1 (Miller *et al.*, 2009) for maximum parsimony, maximum likelihood, and Bayesian analysis. Maximum parsimony was analysed in PAUP 4.0b10 (Swofford, 2003) using a CIPRES wrapper around the PAUP heuristic search command, tree bisection and reconnection branch swapping, and 200 ratchet iterations. Strict and 50% majority rule consensus were calculated to generate a final tree. Maximum likelihood was analysed in RAxML v7.0.4 (Stamatakis, 2006), using the RAxML GTR+G+I model in CIPRES portal v1.15 and with 1000 rapid bootstrap inferences (Stamatakis *et al.*, 2008). Bayesian analysis was conducted in MrBayes v 3.2.1 (Ronquist & Huelsenbeck, 2003), using the GTR+G+I model, the Markov-chain Monte Carlo (MCMC) calculation running for 10,000,000 generations, trees sampled every 1,000 generations, and the first 25% of trees being discarded as burnin. Trees were viewed in Treeview v1.6.6 (Page, 1996).

Microsatellite Markers

Eight SSR loci were successfully amplified using the recommended conditions (Lumley *et al.*, 2009), for 478 Cypress Hills specimens, including the same 477 specimens sequenced for mtDNA. Amplified product was run using an ABI Prism 3730 DNA Analyzer, sized relative to Genescan LIZ-500 (Applied Biosystems, Foster City, CA), then checked and genotyped using GeneMapper

4.0 (Applied Biosystems, Foster City, CA). SSR genotypes from 1135 individuals that were analysed in Chapter 4 were added to the data. These specimens were collected from across North America and included all species within the spruce budworm complex.

SSR data were analysed in Structure v2.3.2 (Pritchard *et al.*, 2000) using the admixture model, with 5 broad geographic regions considered as sampling locality priors: 1) southern British Columbia, southwestern Alberta and the western US; 2) Rocky Mountains (north of Porcupine Hills, AB), northern British Columbia, Yukon, and Alaska; 3) coastal regions of British Columbia and Alaska; 4) east of the Rocky Mountains from Alberta to Newfoundland and the eastern US; and 5) Cypress Hills. Allele frequencies were calculated using both the North American (Chapter 4) and Cypress Hills samples to determine if the Cypress Hills specimens were assigned to the same populations as the North American specimens and thereby were comparable. Allele frequencies were also calculated using the North American samples only, allowing population and individual assignments to be as similar as possible to those completed in Chapter 4, with Cypress Hills specimens assigned to populations based on the calculations for the North American samples. For both analyses, ten iterations for each population size (k) equalling 1 through 13 were analysed with MCMC running for 500,000 generations and initial burnin of 50,000 generations. For the first analysis, with Cypress Hills specimens included in allele frequency calculations, likelihood and ΔK were examined (Evanno *et al.*, 2005) to determine the most likely population size. For the second analysis, with Cypress Hills specimens not included in allele frequency calculations, visual comparisons of the SSR analysis with mtDNA and adaptive traits were completed for k=2 and k=6, since k=2 was the most likely number of populations calculated for the North American samples based on ΔK, and k=6 gave the most resolution for separating described species (Chapter 4).

Morphology

For the pheromone trap collection completed in 2008, the forewings from all captured moths (n=16,440) were examined and classified as brown, grey, or

worn. Morphometric analysis was also performed on 398 specimens that had associated mtDNA and SSR data. These specimens were pinned, photographed and imported into ImageJ 1.38x (Rasband, 2006) to measure 25 morphometric forewing pattern elements as described by Lumley and Sperling (2010). Morphometric measurements were transformed by log base 10 of X + 1 and analysed using linear discriminate analysis in Ginkgo v1.4 (De Cáceres *et al.*, 2003). SSR population (k=2) was the prior method for grouping individuals to determine if these populations could be distinguished using morphology.

Combined data

Data gathered for Cypress Hills specimens were compared visually to data gathered for each of the spruce budworm species collected throughout other regions of North America. This was to determine which species are most likely residing in Cypress Hills. All data types were also compared between specimens collected in Cypress Hills to determine if there were any genetic, morphological, or behavioural traits that differed between but corresponded within population types. This was to determine if there were any characters that may be contributing to the maintenance of the genomic integrity of species or populations. Cypress Hills specimens were also divided into four genetic types based on mtDNA and SSR (k=2) data, these types being: 1) *Eastern*, for f- or p-lineage mtDNA + SSR population 1; 2) *Western*, for o-, oβ- or bβ-lineage mtDNA + SSR population 2; 3) *Intermediate 1*, for o-, oβ- or bβ-lineage mtDNA + SSR population 1; and 4) *Intermediate 2*, for f- or p-lineage mtDNA + SSR population 2. This was to evaluate whether there were any possible hybridization events between the two main populations, as determined by SSR assignment (k=2). For specimens collected in 2008, these four genetic types were also plotted over time to determine if there was an intermediate flight period for hybrids. These genetic types were also mapped onto the LDA morphometric analysis to determine what population they were most similar to morphologically.

Results

Mitochondrial DNA

A total of 165 ingroup haplotypes (Table 5-2; Figure 5-2) were included in the analysis, of which 142 were previously published (Sperling & Hickey, 1994; Roe & Sperling, 2007a; Lumley & Sperling, 2010) or in Chapter 4. These haplotypes represented 1630 individuals, and included all currently known spruce budworm species across their known range as well as the Cypress Hills specimens. From the 477 Cypress Hills specimens sequenced for the 470 bp region of COI mtDNA, a total of 49 ingroup mtDNA haplotypes were found, of which 23 were new and therefore unique to Cypress Hills (previously unpublished haplotypes in Table 5-2).

When restricted to the 470 bp region and with outgroups excluded, 71 characters were parsimony informative, 32 characters were variable but parsimony uninformative, and 367 characters remained constant. Maximum parsimony, maximum likelihood, and Bayesian analysis resulted in trees with similar topologies of major lineages and are in general agreement with those published in other studies (Sperling & Hickey, 1994; Lumley & Sperling, 2010; Chapter 4). The maximum likelihood tree is shown in Figure 5-2. In comparing all locations that I have studied so far, Cypress Hills contains the highest amount of mtDNA diversity, with 49 mtDNA haplotypes and all five major mtDNA lineages (f, o, p, oβ, and bβ) as defined in Sperling & Hickey (1994). The proportion of individuals associated with each lineage differs greatly, with the majority containing the f-lineage (n=241) or o-lineage (n=217) and smaller numbers of individuals containing the p-lineage (n=7), oβ-lineage (n=9) and bβ-lineage (n=3). These lineages are associated with all known spruce budworm species, with the f-lineage primarily associated with *C. fumiferana* and the p-lineage primarily associated with *C. pinus* in other regions, and with the o-, oβ-, and bβ-lineages associated with all western species (*C. occidentalis*, *C. biennis*, *C. orae*, *C. lambertiana*, *C. retiniana*, and *C. carnana*).

Microsatellite Markers

When Cypress Hills specimens were included in the calculation of allele frequencies using Structure (Pritchard *et al.*, 2000), the likelihood values and ΔK (Evanno *et al.*, 2005) indicated that the most likely number of populations is two (Figure 5-2, 5-3). This result was the same as for the North American samples only (Chapter 4), indicating that populations in Cypress Hills could be compared to those in surrounding areas. When Cypress Hills specimens were not included in the calculation of allele frequencies, k=2 indicated that the two populations were both in Cypress Hills (Figure 5-3). Based on the North American samples, Population 1 encompasses species residing in the boreal regions and eastern US (*C. fumiferana*, *C. pinus*) and Population 2 encompasses the remaining species residing in the west (*C. occidentalis*, *C. biennis*, *C. orae*, *C. lambertiana*, *C. retiniana*, *C. carnana*) (Figure 5-3).

I also examined the assignment of Cypress Hills specimens at k=6, as this was the number of populations at which the most North American species were delimited using the eight SSR markers (Chapter 4). In the North American study (Chapter 4), individuals were identified to species on the basis of adaptive traits (life history, behaviour, ecogeography, and morphology), and the SSR analysis grouped these same individuals as Population 1 (*C. fumiferana*), Population 2 (Western A), Population 3 (Western B), Population 4 (*C. lambertiana*), Population 5 (*C. pinus*) and Population 6 (*C. retiniana*). It was determined that Western A and Western B contained the western species, mainly *C. occidentalis*, *C. biennis*, *C. orae*, *C. carnana*, as well as several specimens identified as *C. lambertiana* and *C. retiniana*. Using the same individuals analysed for the North America study (Chapter 4) to assign the Cypress Hills individuals, it was found that Cypress Hills individuals were assigned to Population 1 (*C. fumiferana*, n=269), Population 2 (Western A, n=28), Population 3 (Western B, n=175), Population 4 (*C. lambertiana*, n=4) and Population 5 (*C. pinus*, n=2). No individuals were found that were assigned to Population 6 (*C. retiniana*).

Life-History and Morphology

Life-history, behavioural and morphological data collected for the Cypress Hills specimens were compared qualitatively to data gathered for North American species. Life-history and behavioural data included larval host plant, larval diapause, pheromone attraction, and adult flight phenology. Morphological data included larval head capsule and adult forewing colouration.

The larval host plant for larvae collected in Cypress Hills was almost exclusively spruce (Figure 5-2). Several spruce budworm species are associated with spruce, including *C. fumiferana* (white spruce), *C. biennis* (Engelmann spruce), and *C. orae* (Sitka spruce) (Harvey, 1985). *Choristoneura occidentalis* has also been found to occasionally feed on spruce (Harvey, 1985). Very few larvae (n=3) were found on lodgepole pine despite extensive searching in Cypress Hills. In other regions, *C. lambertiana* is the main species associated with lodgepole pine, although *C. pinus* has also been found to occasionally feed on this host plant (Harvey, 1985). Most Cypress Hills larvae were collected early enough (2^{nd}-4^{th} instar) to determine that they went through only one year of larval diapause. *Choristoneura biennis* larvae go through a second larval diapause, a fixed trait for this species (Nealis, 2005). *Choristoneura orae* larvae may also go through a second larval diapause (Harvey, 1967), and the remaining species typically go through only one year of larval diapause (Harvey, 1985).

Adult *Choristoneura* males were collected in Cypress Hills using both the *C. fumiferana* and *C. pinus* pheromone lures in 2006 and 2008 (Figure 5-2). Counts were only made for the 2008 collection, with a total of 16,210 moths caught using the *C. fumiferana* lure and 224 moths caught with the *C. pinus* lure. Species typically attracted to the *C. fumiferana* lure are *C. fumiferana*, *C. biennis*, *C. occidentalis*, and *C. carnana* (Chapter 4). Species typically attracted to the *C. pinus* lure are *C. pinus*, *C. retiniana*, *C. lambertiana* (Chapter 4), and most likely *C. orae* (Harvey, 1985; unpublished data). Phenology data from 2008 indicate that adults fly from late June to mid September. This covers the flight period of all species within the complex (Freeman, 1967; Powell & De Benedictis, 1995).

For colour pattern, larvae had either dark brown to black (n=184) or lighter brown (n= 33) head capsules (Figure 5-2). In other regions, *C. fumiferana* larvae generally have dark brown to black head capsules, and the remaining species typically have head capsules that are either lighter brown or lighter brown with darker lateral stripes (Harvey & Stehr, 1967; Lumley & Sperling, 2010). Cypress Hills adults had either gray or brown forewings. Species mainly associated with gray forewings are *C. fumiferana*, *C. biennis*, and male *C. orae* (Freeman, 1967). *Choristoneura fumiferana* females and *C. biennis* males and females may also have brown wings (Freeman, 1967). Female *C. orae* , *C. occidentalis*, *C.carnana*, *C. pinus*, and *C. lambertiana* typically have brown forewings (Freeman, 1967). There were no specimens with tawny wings resembling *C. retiniana* (Freeman, 1967).

Morphometrics

Forty-seven morphometric characters were measured for 398 specimens, with SSR population (k=2) as the prior method for grouping individuals, to determine if these two populations could be identified using forewing colour and pattern alone. This also determined which population the intermediate specimens were most similar to in forewing characters. The first canonical discrimination function explained 100% of the variation between the two populations (Figure 5-5) and Wilk's Lambda test of functions was significant ($P<0.05$).

Under no selection, training set resubstitution evaluation and leave-one-out evaluation assigned 352 (88%) and 335 (84%) individuals to the correct population, respectively. Under stepwise selection, the number of characters analysed was reduced to 11 out of the 47 that were measured, and resulted in training set resubstitution evaluation and leave-one-out evaluation correctly identifying 347 (87%) and 342 (86%) individuals to the correct population, respectively. Therefore, the number of characters measured can be decreased without reducing correct identification. As indicated on the graph (Figure 5-5), the two SSR populations form clusters but there is some overlap between them.

Figure 5-5 shows individuals mapped using four different symbols, based on their genetic combination of mtDNA and SSRs (*Eastern*, *Western*, *Intermediate 1*, *Intermediate 2*). This reduced the number of individuals overlapping between the 'pure' populations (*Eastern* and *Western*), though some overlap is still present. It also showed that *Intermediate 1* specimens were clustered with population 1 whereas *Intermediate 2* specimens were more scattered throughout both the population 1 and population 2 clusters.

Combined Data

MtDNA, SSR assignment, life-history, and morphological traits were compared qualitatively as combinations within and between individuals from the Cypress Hills. For the 2008 collections, there were two spruce budworm populations in Cypress Hills that were both attracted to the *C. fumiferana* lure but differed in adult phenology. An early-flying group, collected primarily from June 27 to August 6, were mainly gray-winged moths with f-lineage mtDNA and population 1 SSR assignments (Figure 5-3, 5-4). A late-flying group, collected primarily from August 6 to September 5, were mainly brown-winged moths with o-lineage mtDNA and population 2 SSR assignments (Figure 5-3, 5-4). My data also indicate that there is a third population attracted to *C. pinus* lure. This mid-flying group, collected primarily from July 27 to August 26, is very similar in forewing colour and pattern to the late-flying group, containing mainly brown-winged moths with o-lineage mtDNA and population 2 assignments (Figure 5-3, 5-4).

Comparison of the 2005 larval collections, 2006 adult collections from July 8-11, and 2008 adult collections indicated that the 2005 and 2006 collections were most similar to the 2008 early-flying adults (Figure 5-3). However, there were still a variety of mtDNA haplotypes and assignments to both SSR populations (k=2) within the 2005 and 2006 collections. The 2008 control pheromone traps, containing no pheromone lure, caught a total of six moths: three moths were most likely from the early flying group, having either gray wings or worn wings during the middle of the flight season, f-lineage mtDNA, and

population 1 SSR assignments (k=2); two moths were most likely from the late-flying group, one being brown and the other gray, but both having o-lineage mtDNA and population 2 SSR assignments; and one gray moth was an intermediate, having f-lineage mtDNA but population 2 SSR assignment.

There were rare specimens collected with p-, bβ-, or oβ-lineage mtDNA for which it was difficult to determine whether they were part of the three main populations or represented separate groups. Specimens with p-lineage DNA were collected with *C. pinus* lure within the same time frame as the mid-flying group, and they had similar adult forewing features, population 2 SSR assignment at k=2, and population 2 or 3 at k=6. This differs from the North American samples since specimens with p-lineage mtDNA from east or north of Cypress Hills are highly associated with population 1 at k=2 (Eastern) and population 5 at k=6 (*C. pinus*) (Chapter 4). Specimens with bβ-lineage mtDNA were also collected with the *C. pinus* lure, had a mid-summer flight (July 27 to August 26), were brown-winged, and were assigned to SSR population 2 at k=2 and population 2 or 3 at k=6. Individuals with oβ-lineage mtDNA were collected with the *C. fumiferana* lure, but were mainly late flying (July 17 to September 5) with either brown or gray wings, were assigned to SSR population 1 or 2 (mainly population 2) at k=2, and were assigned to population 1 or 3 (mainly population 3) at k=6.

For the 2008 phenology data, individuals were classified under four genetic types to determine the possibility of hybridization events in Cypress Hills (Figure 5-4). The majority of specimens were either *Eastern* (n= 83) or *Western* (n=182), with the *Eastern* specimens defined by having f- or p-lineage mtDNA and assignment to Population 1 (k=2), and the *Western* specimens defined by having o-, oβ-, or bβ-lineage mtDNA and assignment to Population 2 (k=2). There were also 14 specimens with the *Intermediate 1* genetic type, defined by having o-, oβ-, or bβ-lineage mtDNA and assignment to Population 1 (k=2), and 7 specimens with the *Intermediate 2* genetic type, defined by having f- or p-lineage mtDNA and assignment to Population 2 (k=2). Both *Intermediate 1* and *Intermediate 2*, which may refer to two hybrid genetic types, were mid-summer

fliers in that they were collected as adults from July 7 to August 6. During this time period both the *Eastern* and *Western* populations were flying.

Discussion

Many factors must be taken into account when delimiting and identifying species on islands, including the propensity for species to undergo adaptive radiations and hybridization events in new, geographically constrained ecotypes (e.g. Grant *et al.*, 1996; Schluter, 2000; Seehausen, 2004; Petren *et al.*, 2005). This challenge was faced in identifying spruce budworm individuals to species in Cypress Hills, with the added difficulty that 'mainland' life-history traits or ecogeographical features are typically necessary for species identification. Although isolated, Cypress Hills is situated between two major ecogeographical regions, the boreal and cordilleran, each of which contain different spruce budworm species. Considering the biogeographical history of the region (Strong & Hills, 2005), along with the ability of spruce budworm to migrate long distances (Dobesberger *et al.*, 1983), it is plausible that any of the species in the group occur in Cypress Hills. The documented ability of spruce budworm species to hybridize (Harvey, 1997), adds the possibility of further complexity in the Cypress Hills through secondary contact between previously allopatric species.

By integrating life-history, behaviour, morphology, and genetics I determined that there are probably at least three spruce budworm populations in Cypress Hills, each with a distinct flight period, and each population resembles a 'mainland' species. The early-flying group resembles *C. fumiferana*, with gray forewings, f-lineage mtDNA, and assignment to SSR population 1 (k=6). The late-flying group resembles *C. occidentalis*, with brown forewings, o-lineage mtDNA, and assignment to SSR population 2 or 3 (k=6). Identification of the late-flying group was partly through a process of elimination. No larvae were found in Cypress Hills that went through second diapause and this is a fixed character for *C. biennis* (Nealis, 2005). *Choristoneura carnana*, the remaining species attracted to the *C. fumiferana* lure, has only been found in California and southern Oregon,

which are geographically distant from Cypress Hills. Cypress Hills also contains a smaller midsummer-flying third group that is attracted to the *C. pinus* lure and resembles *C. lambertiana*. All individuals within this group had brown forewings as adults, and most had o-lineage mtDNA and assignment to SSR population 2 or 3.

There were also some individuals that had p- or bβ-lineage mtDNA. Of particular interest, there is evidence for decoupling of p-lineage mtDNA from the usual nuclear genome of *C. pinus*, since SSR population assignment for Cypress Hills specimens containing the p-lineage did not correspond to that of individuals collected in other regions. It is possible that there were previously *C. pinus* individuals residing in Cypress Hills that eventually hybridized with *C. lambertiana* to produce this unusual genotype.

Considering the propensity for species to hybridize in new, geographically confined regions (Seehausen, 2004), as would be the case for Cypress Hills, it is surprising that the two main populations (early- and late-flying) have remained separate with relatively few hybrids, as determined by the proportions of the four mtDNA and SSR combinations (*Eastern*, *Western*, *Intermediate 1*, *Intermediate 2*). Several traits may allow these populations to maintain their genomic integrity. Although these two populations are both attracted to the *C. fumiferana* lure, there may be missing components in the artificial lure that would normally allow individual discrimination. The populations are morphologically different, as determined by simple wing-colour scoring as well as morphometric analysis. Most importantly, they are phenologically different. Although the populations have an overlapping flight period (Figure 5-4A), the number of individuals flying from each population differs substantially, with rapid transition. This may reduce the opportunity for them to hybridize. Interestingly, all identified hybrids had an intermediate flight period that was within the period of time during which both of the main populations were flying. Laboratory experiments on spruce budworm hybrids have also found hybrids to undergo intermediate development between that of their parents, which may result in intermediate flight periods (Smith, 1953; Harvey, 1967; Volney & Liebhold, 1985).

The close and possibly identical resemblance of the Cypress Hills populations to described species found elsewhere in North America indicates that the Cypress Hills populations have not undergone any major divergence or speciation events. One possible explanation for this is based on the work on Caribbean anoles by Losos and Schluter (2000) who found that islands which were smaller than 3,000 km^2 did not host any within-island speciation events. They hypothesized that as the size of an island increases the opportunity for geographic isolation also increases. They also hypothesized that the diversity of habitats increases with geographic area, which allows for increased opportunity for ecological speciation. Therefore, it is possible that the geographic area of Cypress Hills is not large enough to allow opportunity for speciation events. Another possibility is that the Cypress Hills populations have not been isolated for a long enough period of time from their mainland counterparts. This could be due to dispersal events from the mainland regions, or due to an insufficient period of isolation, as it has been hypothesized that Cypress Hills became a forest refugium only 12,000 to 14,000 years before present (Strong & Hills, 2005).

The 2008 early-flying group is more genetically similar to the larvae collected in 2005 than to the 2008 late-flying group. There are three possibilities that may explain this. First, the late-flying group may have migrated from elsewhere, most likely from southern or western regions (e.g. Montana, Idaho, Washington, British Columbia, southern Alberta) where I collected *C. occidentalis* in high numbers and observed severe host defoliation within these same years. Cypress Hills is within the flight range of some of these regions. Second, late-flying individuals may have been feeding in localities or on host plants that were not sampled as larvae. All larvae were sampled from lower, accessible branches of the host plant, so if the late-flyers were feeding in other regions of the host, or residing in areas of Cypress Hills that were not sampled, then they may not have been collected in proportions that represented their numbers as adults. Third, the 2005 larvae were sampled early in the season when they were 2^{nd}-3^{rd} instar so that diapause characteristics could be studied. Late-flying individuals may have still been in diapause or in the process of migrating to

the bud to feed, and therefore may have been missed during collection. This is of particular interest as the late-flying group most resembles *C. occidentalis* and, if resident in Cypress Hills, would have to be adapted to a non-typical host (white spruce or hybrid spruce) that is possibly nutritionally and phenologically different from the typical host (Douglas-fir). Further investigation into these possibilities is a promising avenue for further research.

Overall, my results demonstrate the importance of collecting samples at intervals throughout the overall flight period for studies focused on identifying and monitoring species or populations. This principle applies to biodiversity studies, insect pest and invasive species monitoring, and almost any study focused on systematics or population genetics. Specimens collected in 2005 and 2006, along with prior collections by Sperling & Hickey (1994), indicated that there were different co-occuring mtDNA lineages, but the majority of these specimens resembled *C. fumiferana*. A shortage of specimens and few biological differences made it unreasonable to separate out additional populations. By sampling the full flight period over 10-day intervals in 2008, I was able to determine that there were additional populations with different biological characteristics residing in Cypress Hills that were missed, or misrepresented in proportion, by collecting early, single-period samples (early June for larvae, early July for adults).

This study highlights the importance of using integrative methods and broad sampling for species or population delimitation, a contentious issue amongst taxonomists (e.g. Dayrat, 2005; Will *et al.*, 2005) that is nonetheless increasingly being supported through case-focused research (e.g. Roe & Sperling, 2007b; Ross *et al.*, 2009; Schlick-Steiner *et al.*, 2010). Without combining behavioural, morphological, and genetic traits, further delimitation of the Cypress Hills populations would have remained ambiguous, as they were with the use of mtDNA alone (Sperling & Hickey, 1994). This is particularly true for the identification of closely-related, sympatric species on islands where typical ecogeographical and life-history traits are unavailable.

Table 5-1. General location information for analyzed samples collected in Cypress Hills, Canada.

Locality	Collection Date	Latitude	Longitude	Elevation (m)	Larval Host or Main Tree Species
Larval Collection 2005, 2008					
Canyon Lookout	28.vi.2008	49.635	-110.326	1458	*Picea albertiana*
Firerock	8.vi.2005	49.657	-110.321	1293	*Picea albertiana*
W of Hwy 41	17.vii.2008	49.634	-110.255	1440	*Pinus contorta*
W of Spruce Coulee Rd	7.vii.2008	49.644	-110.228	1441	*Pinus contorta*
S of Reesor Lake Rd	7.vii.2008	49.644	-110.213	1442	*Pinus contorta*
Spruce Coulee	7.vi.2005	49.677	-110.184	1307	*Picea albertiana*
Reesor Lake	7.vi.2005	49.660	-110.099	1291	*Picea albertiana*
Battle Creek	7.vi.2005	49.656	-110.034	1236	*Picea albertiana*
Adult Collection 2006					
Firerock	8.vii-11.vii.2006	49.657	-110.321	1293	Mixed stand
Canyon Lookout	8.vii-11.vii.2006	49.635	-110.326	1458	Mixed stand
Willow Creek	8.vii-11.vii.2006	49.621	-110.299	1416	*Pinus contorta*
Spruce Coulee	8.vii-11.vii.2006	49.677	-110.184	1307	*Picea albertiana*
E of Spruce Coulee Rd	8.vii-11.vii.2006	49.661	-110.185	1415	*Pinus contorta*
Reesor Lake	8.vii-11.vii.2006	49.660	-110.099	1291	*Picea albertiana*
Grayburn	8.vii-11.vii.2006	49.628	-110.051	1381	Mixed stand
Adult Collection 2008					
Firerock	27.vi-5.ix.2008	49.657	-110.321	1293	Mixed stand
Willow Creek	27.vi-5.ix.2008	49.621	-110.299	1416	*Pinus contorta*
Ferguson Hill	27.vi-5.ix.2008	49.637	-110.310	1437	*Pinus contorta*
W of Spruce Coulee Rd	27.vi-5.ix.2008	49.644	-110.228	1441	Mixed stand
E of Spruce Coulee Rd	27.vi-5.ix.2008	49.661	-110.185	1415	*Pinus contorta*
Spruce Coulee	27.vi-5.ix.2008	49.677	-110.184	1307	*Picea glauca*
Reesor Lake	27.vi-5.ix.2008	49.660	-110.099	1291	*Picea glauca*
Grayburn	27.vi-5.ix.2008	49.628	-110.051	1381	Mixed stand
Battle Creek	27.vi-5.ix.2008	49.656	-110.034	1236	*Picea glauca*

Table 5-2. MtDNA haplotypes with corresponding GenBank accession numbers and references.

Haplotype	Accession No.	References
f1	L19098[a]	Sperling & Hickey, 1994; Roe & Sperling, 2007; Lumley & Sperling, 2010; Chapter 4
f2	GQ890278[a]	Sperling & Hickey, 1994; Roe & Sperling, 2007; Lumley & Sperling, 2010; Chapter 4
f3	GQ890279	Sperling & Hickey, 1994; Lumley & Sperling, 2010; Chapter 4
f4	HM223075	Chapter 4
f5	HM223076	Chapter 4
f6	HM223077	Previously unpublished
f7	HM223078	Chapter 4
f8	HM223079	Previously unpublished
f9	GQ890280	Lumley & Sperling, 2010; Chapter 4
f10	GQ890281	Lumley & Sperling, 2010; Chapter 4
f11	GQ890282	Lumley & Sperling, 2010; Chapter 4
f12	HM223080	Previously unpublished
f13	HM223081	Chapter 4
f14	HM223082	Previously unpublished
f15	HM223083	Chapter 4
f16	HM223084	Previously unpublished
f17	GQ890283	Lumley & Sperling, 2010; Chapter 4
f18	HM223085	Previously unpublished
f19	HM223086	Previously unpublished
f20	HM223087	Previously unpublished
f21	HM223088	Previously unpublished
f22	HM223089	Previously unpublished
f23	HM223090	Previously unpublished
f24	HM223091	Previously unpublished
f25	HM223092	Chapter 4
f26	HM223093	Chapter 4
f27	HM223094	Chapter 4
f28	HM223095	Chapter 4
f29	HM223096	Chapter 4
f30	HM223097	Chapter 4
f31	HM223098	Chapter 4
f32	HM223099	Chapter 4
f33	HM223100	Chapter 4
f34	HM223101	Chapter 4
p1	L19095[a]	Sperling & Hickey, 1994; Roe & Sperling, 2007; Lumley & Sperling, 2010; Chapter 4
p2	HM223102	Chapter 4
p3	GQ890284	Lumley & Sperling, 2010; Chapter 4
p4	GQ890285	Lumley & Sperling, 2010; Chapter 4
p5	GQ890286	Lumley & Sperling, 2010; Chapter 4
p6	GQ890287	Lumley & Sperling, 2010; Chapter 4
p7	GQ890288	Lumley & Sperling, 2010; Chapter 4
p8	GQ890289	Lumley & Sperling, 2010; Chapter 4
p9	HM223103	Chapter 4
p10	HM223104	Chapter 4
p11	HM223105	Chapter 4
p12	HM223106	Chapter 4
p13	HM223107	Chapter 4
o1	L19094, DQ792584[a]	Sperling & Hickey, 1994; Roe & Sperling, 2007; Lumley & Sperling, 2010; Chapter 4
o2	GQ890290	Sperling & Hickey, 1994; Lumley & Sperling, 2010; Chapter 4
o3	HM223108	Chapter 4
o4	HM223109	Chapter 4
o5	GQ890291	Sperling & Hickey, 1994; Lumley & Sperling, 2010; Chapter 4
o6	HM223110	Chapter 4
o7	HM223111	Chapter 4
o8	HM223112	Chapter 4
o9	HM223113	Chapter 4
o10	HM223114	Previously unpublished
o11	GQ890292	Lumley & Sperling, 2010; Chapter 4
o12	HM223115	Previously unpublished
o13	HM223116	Chapter 4

Table 5-2. cont.

Haplotype	Accession No.	References
o16	HM223119	Previously unpublished
o17	HM223120	Chapter 4
o18	HM223121	Chapter 4
o19	HM223122	Previously unpublished
o20	HM223123	Chapter 4
o21	HM223124	Previously unpublished
o22	HM223125	Previously unpublished
o23	HM223126	Previously unpublished
o24	HM223127	Chapter 4
o25	HM223128	Chapter 4
o26	HM223129	Previously unpublished
o27	HM223130	Previously unpublished
o28	HM223131	Chapter 4
o29	HM223132	Previously unpublished
o30	HM223133	Chapter 4
o31	HM223134	Chapter 4
o32	HM223135	Chapter 4
o33	HM223136	Chapter 4
o34	HM223137	Chapter 4
o35	HM223138	Chapter 4
o36	HM223139	Chapter 4
o37	HM223140	Chapter 4
o38	HM223141	Chapter 4
o39	HM223142	Chapter 4
o40	HM223143	Chapter 4
o41	HM223144	Chapter 4
o42	HM223145	Chapter 4
o43	HM223146	Chapter 4
o44	HM223147	Chapter 4
o45	HM223148	Chapter 4
o46	HM223149	Chapter 4
o47	HM223150	Chapter 4
o48	HM223151	Chapter 4
o49	HM223152	Chapter 4
o50	HM223153	Chapter 4
o51	HM223154	Chapter 4
o52	HM223155	Chapter 4
o53	HM223156	Chapter 4
o54	HM223157	Chapter 4
o55	HM223158	Chapter 4
o56	HM223159	Chapter 4
o57	HM223160	Chapter 4
o58	HM223161	Chapter 4
o59	HM223162	Chapter 4
o60	HM223163	Chapter 4
o61	HM223164	Chapter 4
o62	HM223165	Chapter 4
o63	HM223166	Chapter 4
o64	HM223167	Chapter 4
o65	HM223168	Chapter 4
o66	HM223169	Chapter 4
o67	HM223170	Chapter 4
o68	HM223171	Chapter 4
o69	HM223172	Chapter 4
o70	HM223173	Chapter 4
o71	HM223174	Chapter 4
o72	HM223175	Chapter 4
o73	HM223176	Chapter 4

Table 5-2. cont.

Haplotype	Accession No.	References
o76	HM223179	Chapter 4
o77	HM223180	Chapter 4
o78	HM223181	Chapter 4
o79	HM223182	Chapter 4
o80	HM223183	Chapter 4
o81	HM223184	Chapter 4
o82	HM223185	Chapter 4
o83	HM223186	Chapter 4
o84	HM223187	Chapter 4
o85	HM223188	Chapter 4
o86	HM223189	Chapter 4
o87	HM223190	Chapter 4
o88	HM223191	Chapter 4
o89	HM223192	Chapter 4
o90	HM223193	Chapter 4
o91	HM223194	Chapter 4
o92	HM223195	Chapter 4
o93	HM223196	Chapter 4
b1	DQ792586, DQ792587[a]	Sperling & Hickey, 1994; Roe & Sperling, 2007; Lumley & Sperling, 2010; Chapter 4
b2	HM223197	Chapter 4
b3	HM223198	Chapter 4
oB	DQ792585[a]	Sperling & Hickey, 1994; Roe & Sperling, 2007; Chapter 4
oB2	HM223199	Chapter 4
oB3	HM223200	Chapter 4
oB4	HM223201	Chapter 4
oB5	HM223202	Chapter 4
oB6	HM223203	Chapter 4
oB7	HM223204	Chapter 4
oB8	HM223205	Chapter 4
oB9	HM223206	Chapter 4
oB10	HM223217[a]	Chapter 4
oB11	HM223207	Chapter 4
oB12	HM223208	Chapter 4
bB	L19096[a], L19097	Sperling & Hickey, 1994; Roe & Sperling, 2007; Chapter 4
bB2	HM223209	Chapter 4
bB3	HM223210	Chapter 4
bB4	HM223211	Chapter 4
bB5	HM223212	Chapter 4
bB6	HM223213	Chapter 4
bB7	HM223214	Chapter 4
bB8	HM223218[a]	Chapter 4
bB9	HM223215	Chapter 4
bB10	HM223216	Chapter 4
c1	GQ890293[a]	Lumley & Sperling, 2010; Chapter 4
m1	GQ890294[a]	Lumley & Sperling, 2010; Chapter 4
r1	L19099[a]	Sperling & Hickey, 1994; Chapter 4
r2	GQ890295	Sperling & Hickey, 1994; Lumley & Sperling, 2010; Chapter 4

[a] Sequence analyzed for 2.3 kb COI and COII region

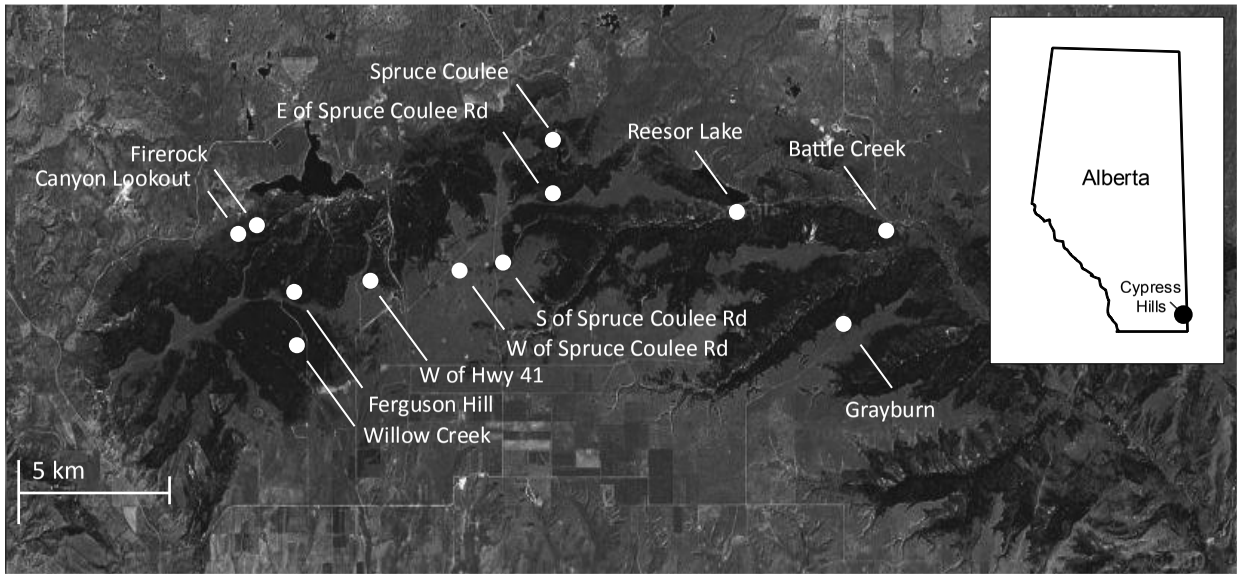

Figure 5-1. Cypress Hills collection locations from which specimens were sampled and analyzed. Photo source: Google Maps (http://maps.google.com/)

Following Page:

Figure 5-2. Maximum likelihood tree for 165 unique ingroup haplotypes found in the *Choristoneura fumiferana* species complex. The analysis included the full 2.3 kb region of COI and COII mitochondrial DNA for 12 individuals and a 470 bp region of COI mitochondrial DNA for 1632 individuals. Maximum likelihood bootstrap values and Bayesian support values are indicated for the main lineages. Haplotypes labelled by an asterix (*) were found in Cypress Hills. Beside each haplotype found in Cypress Hills is phenotype information for specimens containing that haplotype (Cypress Hills specimens only), including: A) number of individuals containing the haplotype; B) larval head colour; C) larval host plant; D) adult wing colour; E) pheromone attraction; F) the main tree species in the locality where the specimens were collected; G) adult phenology for 2008 pheromone trap collection; H) SSR assignment for k=2; and I) SSR assignment for k=6.

Figure 5-2. See previous pg. for figure caption

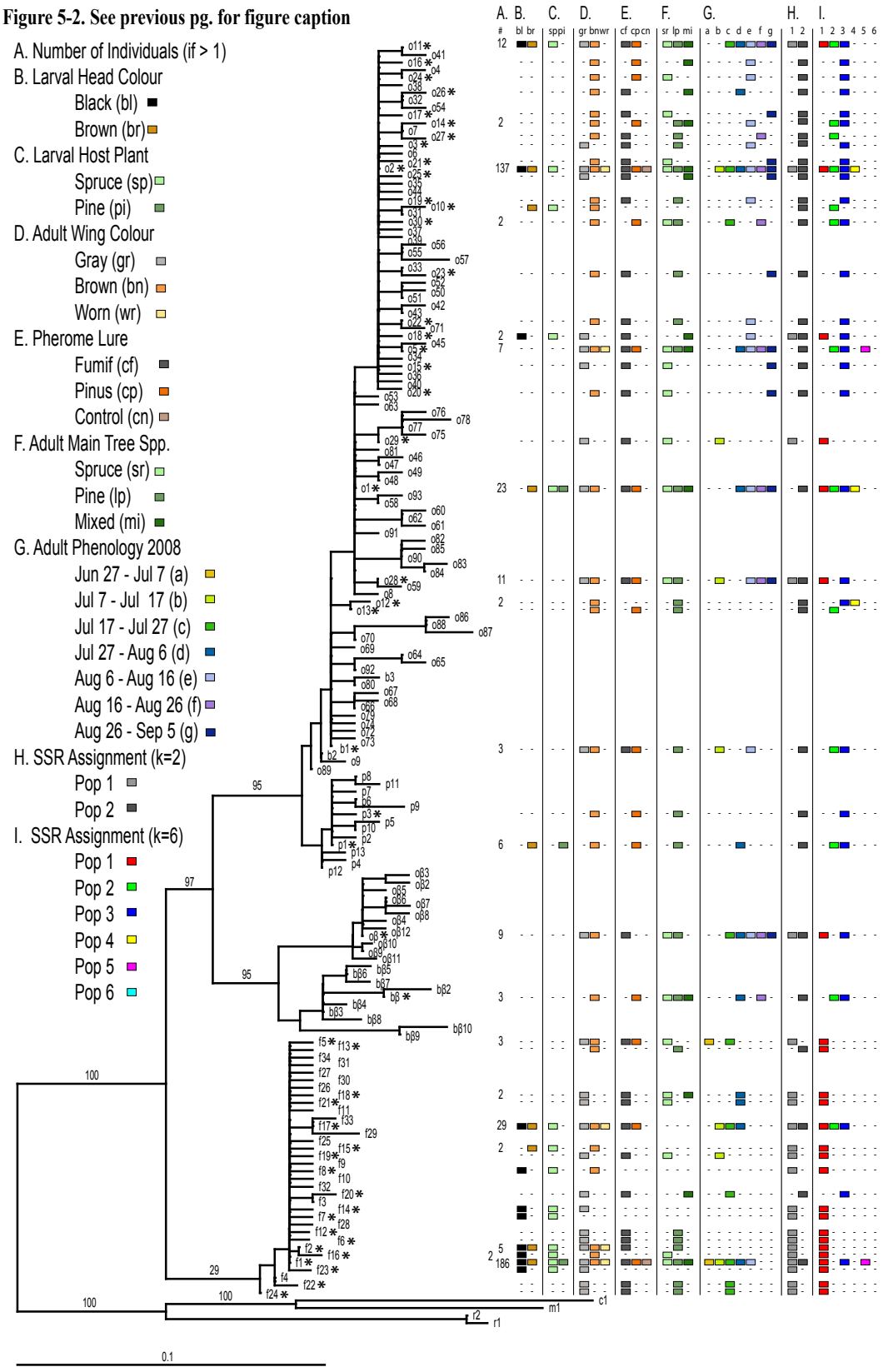

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Figure 5-3. Probability of simple sequence repeat assignment using Structure analysis (k=2), with North American samples (excluding all but 10 Cypress Hills specimens, as per Chapter 4) grouped by species, and Cypress Hills samples grouped by collection information (collection year, collection taken as larvae or adults, larval host plant, adult trap type, and collection date for 2008 phenology study). Abbreviations: *lamb* = *lambertiana*; *retin* = *retiniana*; *carn* = *carnana*; spr = spruce; pi = lodgepole pine; *F* lure = *C. fumiferana* lure; *P* lure = *C. pinus* lure; BL = blacklight; Ct = Control; a-g = collection dates (a = 27 June-7 July; b = 7-17 July; c = 17-27 July; d = 27 July-6 August; e = 6-16 August; f = 16-26 August; g = 26 August-5 September).

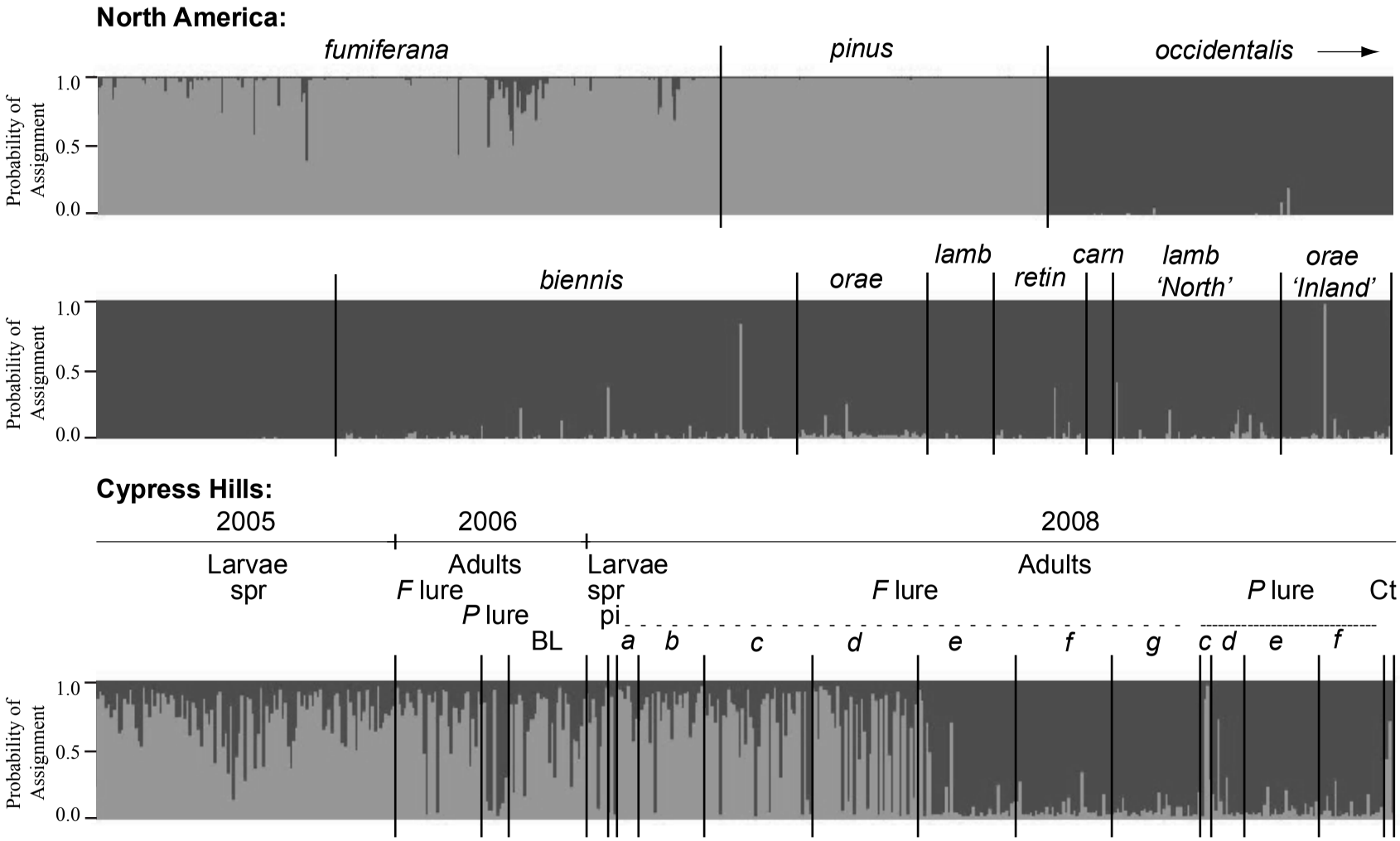

Figure 5-3. See previous page for figure caption.

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Figure 5-4. Phenology histograms for the 2008 adult samples, showing correlation with: A1-3. wing colouration, separated by adult pheromone lure type; B. mtDNA lineage; C. SSR assignment (k=2); D. mtDNA and SSR assignment combined, giving four genetic combinations: Eastern = f- or p-lineage mtDNA + SSR population 1; Western = o-, oβ- or bβ-lineage mtDNA + SSR population 2; Intermediate 1 = o-, oβ- or bβ-lineage mtDNA + SSR population 1; and Intermediate 2 = f- or p-lineage mtDNA + SSR population 2. Letters a-g = collection dates (a = 27 June-7 July; b = 7-17 July; c = 17-27 July; d = 27 July-6 August; e = 6-16 August; f = 16-26 August; g = 26 August-5 September).

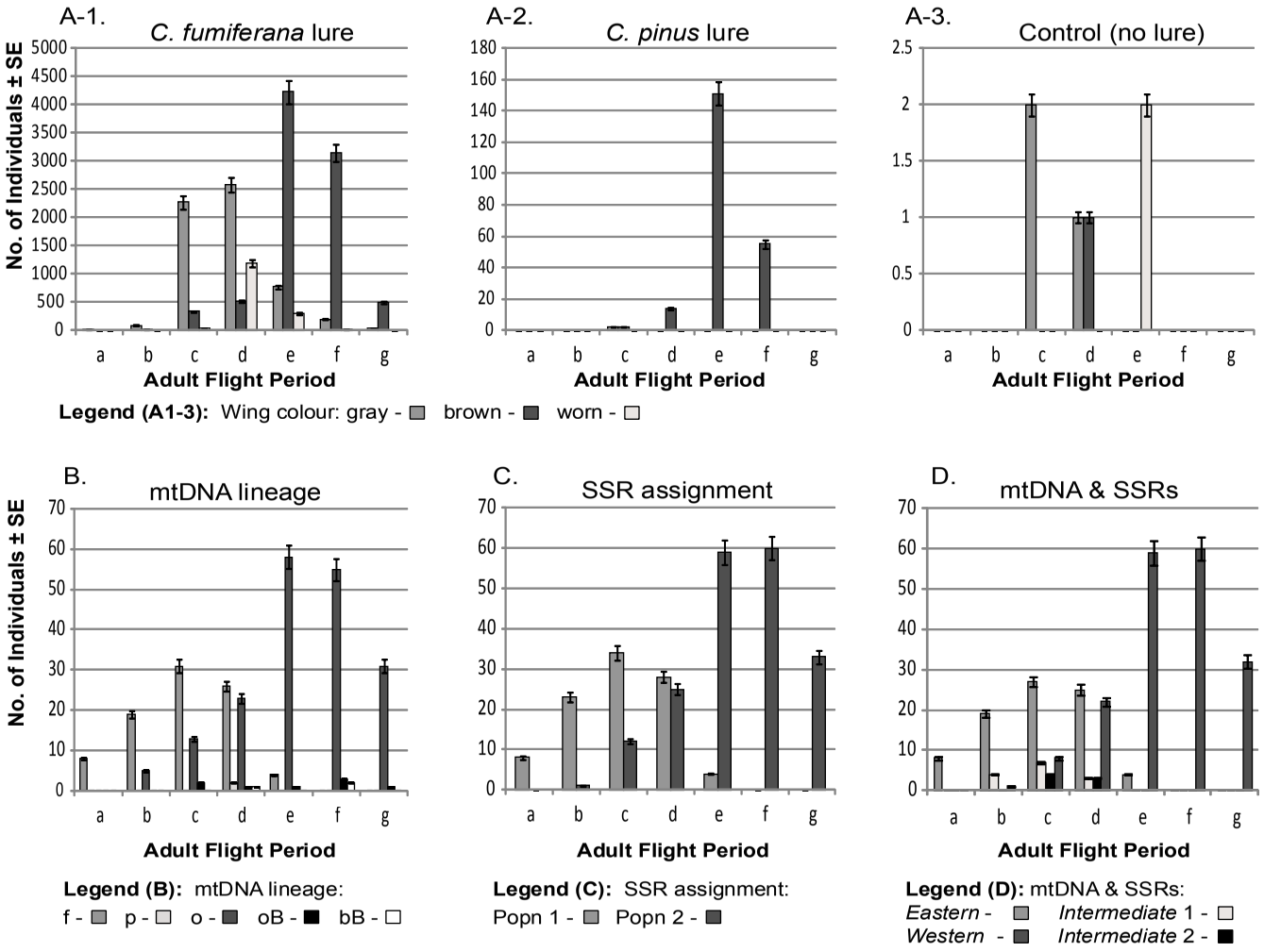

Figure 5-4. See previous page for figure caption.

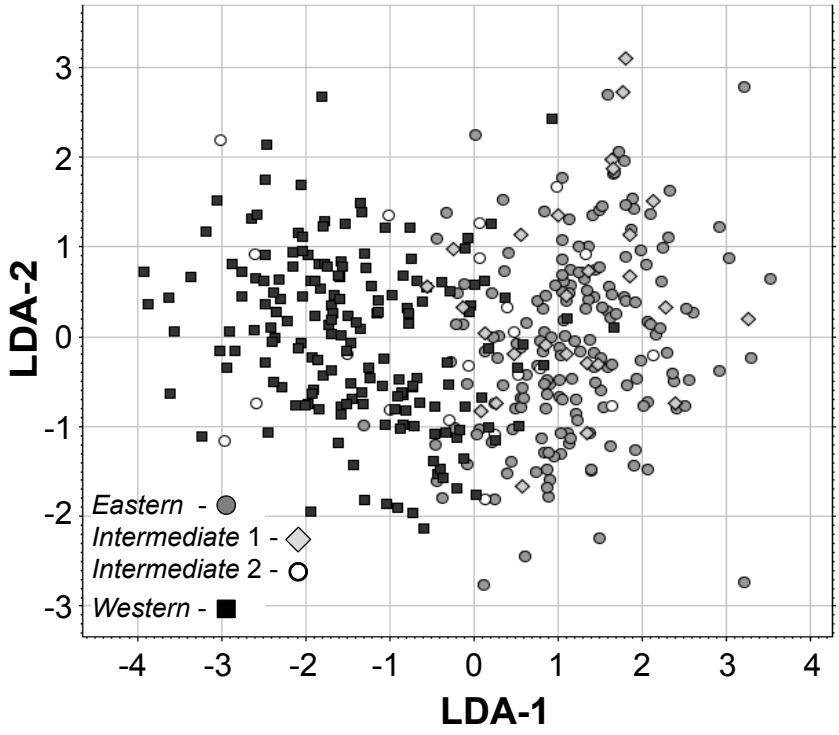

Figure 5-5. Linear discriminant analysis of 47 morphometric characters for Cypress Hills specimens, grouped a priori by SSR population (k=2). Specimen coordinates are labelled to indicate the four genetic combinations: Eastern = f- or p-lineage mtDNA + SSR population 1; Western = o-, oβ- or bβ-lineage mtDNA + SSR population 2; *Intermediate* 1 = o-, oβ- or bβ-lineage mtDNA + SSR population 1; and *Intermediate* 2 = f- or p-lineage mtDNA + SSR population 2.

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Chapter 6

General Conclusions

Thesis Summary

The main objectives of my thesis were to find reliable characters that could be used to identify and delimit species in the spruce budworm (*Choristoneura fumiferana*) complex, identify gene flow or possible hybridization events between putative species, and identify possible mechanisms that may allow species to maintain their genomic integrity even though they have the ability to hybridize and produce viable offspring.

Species delimitation has historically been a challenge for spruce budworms, due to their identifying characters being polymorphic both within and between species (Nealis, 2008). Along with collecting the kind of information in the field that is typically needed for species identification, I examined morphometric variation (Chapter 2), mitochondrial DNA (mtDNA) (Chapter 2, 4), and simple sequence repeats (SSRs, also referred to as microsatellite markers) (Chapter 3, 4) to determine their usefulness for identification of species within the complex. I also conducted a finer-grained study in Cypress Hills (Chapter 5) to determine if the above methods could be used to identify species units in a region where ecogeographical traits are non-typical, and where the possibility of sympatric species was suggested by a previous study conducted by Sperling and Hickey (1994).

Overall, morphometrics proved to be useful for the five species tested in Alberta and south-western British Columbia, with only *C. fumiferana* and *C. biennis* overlapping slightly, whereas the remaining three species (*C. pinus*, *C. occidentalis*, *C. lambertiana*) formed separate clusters based on quantitative wing colouration characteristics (Chapter 2). I found that mtDNA was not very effective for delimiting species. Only two mtDNA lineages were species-specific, these being the f-lineage for *C. fumiferana* and the p-lineage for *C. pinus*, whereas the remaining species shared haplotypes (Chapter 2, 4). SSRs were more effective

than mtDNA, since four species separated into relatively discrete clusters (*C. fumiferana*, *C. pinus*, *C. lambertiana*, *C. retiniana*). However, the remaining species were still mixed within two SSR populations (Chapter 4). Neither of the genetic methods was very helpful at the subspecies level, with only *C. pinus maritima* seeming to have a unique character combination, being bβ-lineage mtDNA and microsatellite population 5. However, so few specimens of this subspecies were available that this result must be deemed preliminary.

Typically, mtDNA and SSRs are considered neutral and they are useful for measuring gene flow. Since neither of these characters was fully effective for delimiting the western species, this result suggests that these species are either in the midst of speciating, or have speciated but still have some gene flow occurring among them. In turn, if we consider the results from the morphometrics (Chapter 2), as well as other collected field and lab data (larval and adult morphology, length of larval diapause, larval host association, pheromone attraction) (Chapter 2, 4), there are putative adaptive traits that are different between species. This indicates that these entities are real, even though we have not found a genetic method to consistently identify them. Therefore, adaptive markers are still necessary for species identification. These results also suggest that developing adaptive genetic markers for spruce budworm species identification may be a promising avenue for further research.

The above character types (mtDNA, SSRs, morphometrics and other adaptive traits) proved useful in resolving species in Cypress Hills, where I conducted a finer-grained study to identify which species had colonized or been retained on this isolated forest island (Chapter 5). They were used in an integrative fashion along with adult flight phenology to determine that there were at least three populations, resembling *C. fumiferana*, *C. occidentalis*, and *C. lambertiana*. This would not have been possible without extensive sampling at regular time intervals, since adult flight phenology was an essential factor in identification of the different groups. Genetic, morphological, ecological, and behavioural data that had been recorded as being associated with species in other forested regions of North America (Chapter 4), were also essential as they

allowed me to assign Cypress Hills individuals to putative species based on 'mainland' traits. Phenology was also identified as a possible mechanism for the maintainance of species units. In more general terms, Chapter 5 explored techniques for delimiting species in regions where they are unknown, and may be a helpful guide to follow for delimiting species on islands or in regions where species are sympatric or parapatric.

Future Directions

There are many threads to follow from the results of my thesis. Some of these can be followed up on immediately, whereas others are hopeful future endeavours. Several additional studies could be started with no additional field or lab work, based on the immense amount of ecological, behavioural, morphological, and genetic data that I have compiled over the last few years. First, the sequence data will allow me to do coalescence analysis which could be used to explore the phylogeography of the species complex. I am interested in combining the genetic and ecological data for a landscape genetics approach at different levels (species, species pairs, pheromone groups, spruce-feeding & pine-feeding groups, and the entire complex). I am also interested in exploring different tree methods for quantitative data (morphometrics) and SSRs to determine if there is any resolution in the spruce budworm phylogeny using alternate phylogenetic techniques. The morphometrics system developed in Chapter 2 should also be explored to determine if it is useful to identify all species in the complex across their known geographic range.

Over the longer term, I hope that the results from my thesis will lead to new lines of research and generate productive future collaborations. Of particular interest would be to associate adaptive traits to genetic markers in the spruce budworm complex and then develop these genetic markers to identify species. Also, I would be very interested in returning to regions where species could not be determined with confidence (e.g. *Choristoneura 'Inland'*, *Choristoneura lambertiana 'North'*) and use the Cypress Hills chapter (Chapter 5) as a model to

identify these phenotypes to species. Using the Cypress Hills chapter as a model in regions where species or genetic types are overlapping may help us to determine if, or how, species are maintaining their genomic integrity. This would also allow us to more confidently determine which adaptive traits to target for adaptive genetic marker development. In addition, if the morphometrics approach proves useful across a larger geographical range and for more species, then research into the development of an automated system for identifying species using this approach would make it more practical for general use.

In conclusion, the spruce budworm species complex still holds many mysteries that will take several life-times to resolve, if not more. However, continued work will allow us to gain further insights into mechanisms of speciation, and further the development of appropriate methods for delimiting closely related species groups.

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Biography

On January 2, 1977, I was born in the Vermilion hospital to Margaret & Elmer Lumley, and became the youngest of four children. I grew up on a grain and cattle farm east of Vermilion. Along with spending time with the 'mega-fauna' ('pail-bunter' calves, horses, sheep, pigs, chickens, turkeys, ducks, cats, rabbits, and dogs), I spent a lot of time exploring the sloughs and pastures, coming home with butterflies and grasshoppers, and 'frog eggs' that turned into mosquitoes. I loved gardening, sewing, horseback riding, reading, and collecting things (shells, spoons, rocks, stamps, coins). Additional extracurricular activities included basketball, volleyball, and piano lessons.

In 1995, I started my B.Sc. in Agriculture majoring in crop and horticultural sciences. During my degree, I had the opportunity to work during a summer for the East Central Alberta Forage Association. My supervisor, Agnes Whiting, introduced me to the technical aspects of crop scouting for bertha armyworm, diamondback moth, and wheat midge. This led into a summer position with United Grain Growers, where I scouted for insects, weeds, and diseases in field crops. Then came a contract position with Integrated Crop Management Services, where my job description included managing the insect trials. My interest in entomology continued to grow through these experiences.

After a four month backpacking trip through South America, I was employed from 2000-2002 as a research technician with the Alberta Research Council. I was in the entomology lab, with Ken Fry as my supervisor. At first, I mainly worked on a field-trial baculovirus experiment on bertha armyworm. Then, I focused on a biodiversity study in canola where I slogged through canola fields to collect insects and trap small mammals. The winter was spent sorting >300,000 insects! During this time I also went on a trip to Belize where I got my diving certification, adding an amazing new dimension to life.

In 2002, I took on a position at the Vermilion Credit Union as an Agricultural Account Manager, where I maintained an agricultural and commercial loan portfolio. On one of my farm calls in 2003, a farmer showed me

dead grasshoppers that were clinging to the tops of the wheat and flax stems and it made me realize how much I missed the bugs! So I decided to go back to university and continue my education as an entomologist. I finished my position at the Credit Union in March 2004, and then went travelling for two months to Southeast Asia before working for the summer for Felix Sperling as a technician.

I started my MSc in Felix Sperling's lab at the University of Alberta in September 2004, and then switched to a PhD in 2006. During this time I have had many amazing field experiences, learned many new technical skills, and expanded conceptually. I am excited for whatever opportunities in entomology may await me!