Temporal isolation and genetic divergence in the *Choristoneura fumiferana* species complex

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Systematics and Evolution

Department of Biological Sciences
University of Alberta

Abstract

Temporal isolation contributes to ecological speciation in a diversity of insect taxa. Such prezygotic isolation can reduce or stop hybridization between closely related taxa or populations, leading to speciation. Within the spruce budworm (*Choristoneura fumiferana*) species complex, hybridization between species occurs freely in laboratory settings but hybrids are rarely found in the wild. Temporal isolation has been suggested as a mechanism that reduces their hybridization, but this has not been assessed for members of the complex that interact in west-central Alberta and adjacent British Columbia. I sought to determine whether temporal isolation reduces hybridization between *C. fumiferana* and *C. occidentalis biennis*, using ddRADseq to genotype 261 individual *Choristoneura* collected over two years. I found that *C. fumiferana* and *C. o. biennis* have significantly different flight peaks, partially due to post-diapause degree-day requirements. However, adults of these taxa contact each other with sufficient frequency to allow hybridization at an estimated rate of 2.9%. I did not find significant genetic differentiation between the even- and odd-year cohorts of *C. o. biennis*. Additionally, collections of *C. o. biennis* extended at least to Elk Island National Park in central Alberta, which is more than 100 km further east than previously recognized. In conclusion, temporal isolation likely contributes to the maintenance of genomic integrity between species of spruce budworm, but the role of other mechanisms should also be investigated.

Acknowledgements

The path taken while completing a graduate degree is not one that is travelled alone. I have many individuals to thank, but the constant overseer of this project has been my supervisor, Felix Sperling. Without your guidance, I would not have become the bug nerd that I am today, nor would I have had the opportunity to spend quite so much time discovering Alberta and its wonderful Lepidoptera (including the little brown moths!)

There are numerous members of the Sperling Lab, both past and present, who deserve more than a simple thanks. Zac MacDonald, Erin Campbell, Brittany Wingert, Victor Shegelski, Rowan French, Janet Sperling, Stephen Trevoy, Bryan Brunet, Lisa Lumley, Gwylim Blackburn, Giovanny Fagua, Pasan Lebunasin Arachchige, Julian Dupuis, Heather Leibel, Kyle Snape, Marnie Wright, Christianne Nylund, and Sarah Leo have each made contributions to this project that cannot be explained in the meager space offered here. Thank you, everyone.

Of course, there were many others who I met along the way who each contributed to the completion of the degree. Mike Kokich, Andrew Cook, Nick Grocock, Ronald Batallas, Caroline Whitehouse, Jonathan Backs, Kelsey Jones, Maggie MacDonald, Antonia Musso, Dylan Sjolie, Valerie Marshall, Federico Riva, Oksana Vernygora, Mónica Higuera, Ashton Sturm, Monica Kohler, and Angela Phung each had valuable input during the project. To the research scientists and professors that set me on the path to this graduate degree, notably Jon Sweeney, Jessamyn Manson, Kirk Hillier, and Deb Moreau, I thank you. To members of the Alberta Lepidopterists' Guild, namely Charley Bird, Greg Pohl, Dave "Fizzy D" Lawrie, Gerry "Blue Wizard" Hilchie, John Acorn, Gary Anweiler, Loney Dickson, Vic Romanyshyn, Hilary Pittel, Bruce Christensen, Bob Parsons, Colleen Raymond, and Gerald Romanchuk, thank you for allowing a junior butterfly enthusiast among your ranks.

I would also like to thank the members of my committee, Maya Evenden and Carol Frost. Your hard work is appreciated, as is your willingness to participate in a thesis defence in early August. Maya, thank you for allowing me to borrow your lab's Unitraps, hexane, and HOBO data loggers. This project would not have happened without your generous contributions.

I must also thank the 8,289 spruce budworms that dutifully gave their lives for the good of this thesis. Your SNPs will be used in future projects to help us understand you.

Finally, my family deserves the utmost thanks for their love and support during the completion of this degree. Hayley, Mum, and Dad – I am grateful for each of you. And Al, thanks for being you. I can't imagine I would have made it through this degree had we not been together in Edmonton.

This project was funded by a Natural Sciences and Engineering Research Council of Canada Discovery Grant awarded to Felix Sperling. Collection permits were granted by the Alberta Provincial government (Permits 17-123, 18-598). Roger Lévesque and Michel Cusson provided access to the *Choristoneura fumiferana* draft reference genome to which DNA sequences were aligned.

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List of Abbreviations

BAM – binary alignment map

bie – Choristoneura occidentalis biennis

BLASTX – basic alignment and search tool (translated nucleotide to protein)

bp – base pairs

ddRADseq – double-digest restriction-site associated deoxyribose nucleic acid sequencing

DNA – deoxyribose nucleic acid

F1 – first filial generation

F2 – second filial generation

fum – Choristoneura fumiferana

kb – kilobases (1000 base pairs)

mtDNA – mitochondrial deoxyribose nucleic acid

NCBI – National Centre for Biotechnology Information

occ – Choristoneura occidentalis occidentalis

PC1 – principal component axis one

PC2 – principal component axis two

PCA – principal component analysis

SAM – sequence alignment map

SNP – single nucleotide polymorphism

UASM – University of Alberta Strickland Museum

VCF – variant call format

Chapter 1

Introduction and Thesis Objectives

General Introduction

Biologists have long recognized that ecological factors contribute to speciation (e.g. Walsh 1864; Mayr 1947; Bush 1969). However, research on "ecological speciation", which is ecologically-based divergent selection that arrests gene flow between populations (Nosil 2012), has become a major theme in evolutionary research through the combined analysis of molecular and ecological data. Ecological speciation requires reproductive isolation (Rundle & Nosil 2005), which can be provided by time-based isolation of reproductive adults within or between populations, often called temporal isolation (Drès & Mallet 2002; Hendry & Day 2005; Taylor & Friesen 2017). The most widely known exemplar of temporal isolation may be the periodical cicadas; 17-year cicadas are isolated from their 13-year relatives by a four-year period of developmental delay that only allows them to contact each other every 221 years (White & Lloyd 1975; Lloyd & White 1976; Marshall & Cooley 2000). Other examples are found in a diversity of organisms, such as flowering plants (reviewed in Lowry, Modliszewski, Wright, Wu, & Willis 2008), fungi (Kiss *et al.* 2011), corals (Levitan *et al.* 2004), salmon (Quinn, Unwin, & Kinnison 2000), seabirds (Friesen *et al.* 2007), and other insects (Boumans, Hogner, Brittain, & Johnsen 2017; Amari *et al.* 2018).

The spruce budworm species complex comprises eight or nine species of moths that interact across North America (Lumley & Sperling 2011a; Brunet *et al.* 2017; Dupuis *et al*. 2017). They have been the subject of many studies due to their economic importance (Harvey 1985; Volney & Fleming 2007; Nealis 2016), but their interactions remain poorly understood.

There is ample evidence that different species within the complex can mate and produce fertile offspring in experimental crosses (Smith 1953; Campbell 1967; Sanders, Daterman, & Ennis 1977; Harvey & Roden 1979; Liebhold, Volney, & Waters 1984; Volney, Liebhold, & Waters 1984; Liebhold 1986; Harvey 1997; Nealis 2005), and some naturally occurring hybrid individuals have been found (Harvey & Roden 1979; Liebhold *et al.* 1984; Volney *et al.* 1984; Sperling & Hickey 1994; Powell & De Benedictus 1995; Shepherd, Gray, & Harvey 1995; DeVerno, Smith, & Harrison 1998; Lumley & Sperling 2011a, 2011b; Brunet, Hundsdoerfer, & Sperling 2013; Brunet *et al.* 2017; Blackburn *et al.* 2017).

However, natural hybrids have only been collected in low frequencies (Lumley & Sperling 2011b; Brunet *et al.* 2013; Brunet 2014; Brunet *et al*. 2017). This paucity of interspecific hybrid adults in the complex suggests the potential for isolating mechanisms, ranging from premating isolation to postzygotic incompatibility, that may contribute to maintaining the genomic integrity of the species. There is some evidence that seasonal temporal isolation arrests gene flow between spruce budworm species (Smith 1953, 1954; Lumley & Sperling 2011b), although it is weak in some interactions (Volney, Waters, Akers, & Liebhold 1983; Liebhold & Volney 1984; Volney & Liebhold 1985; Powell & De Benedictus 1995). This thesis is focused on evaluating seasonal temporal isolation among conifer-feeding spruce budworm species along the Canadian Rocky Mountains.

The Spruce Budworm (*Choristoneura fumiferana*) Species Complex

Spruce budworms form a species complex that includes several coniferophagous insect pests of major economic importance in North America. They are widespread across the northern and western regions of the continent and have been described as the most destructive insect

defoliators of forests in North America (Volney & Fleming 2007). The species of greatest economic importance, *Choristoneura fumiferana* (Clemens 1865), is known as *the* spruce budworm and ranges in boreal forests from the Atlantic provinces of Canada west to the Rocky Mountains and Alaska (Harvey 1985; Lumley & Sperling 2011a). Its larvae primarily feed on white spruce, *Picea glauca* (Moench) Voss, and balsam fir, *Abies balsamea* (L.) Mill. (Shepherd *et al.* 1995).

In the foothills of west-central Alberta, where boreal forest meets the Rocky Mountains, the range of *C. fumiferana* overlaps with several other spruce budworm taxa (Lumley & Sperling 2011a; Brunet *et al.* 2017), particularly the two-year-cycle spruce budworm, *Choristoneura occidentalis biennis* Freeman 1967. In southern Alberta, *C. fumiferana* also contacts the western spruce budworm, *Choristoneura occidentalis occidentalis* Freeman 1967 (Lumley & Sperling 2011a; Brunet *et al.* 2013). Larvae of *C. o. biennis* primarily feed on subalpine fir, *Abies lasiocarpa* (Hook.) Nuttall, and Engelmann spruce, *Picea engelmannii* Parry ex. Engelmann (Shepherd *et al.* 1995), whereas larvae of *C. o. occidentalis* primarily feed on Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco (Shepherd *et al*. 1995). The geographic range of each is closely associated with its primary host plant (Volney & Cerezke 1992; Lawrence, Mattson, & Haack 1997; Nealis 2012; Fuentealba, Pureswaran, Bauce, & Despland 2017).

Taxa in the spruce budworm species complex have been identified with varying success using morphology (MacKay 1953, 1962; Freeman 1967; Harvey & Stehr 1967; Volney *et al.* 1984; Dang 1985, 1992; Liebhold 1986; Gray, Shepherd, & Harvey 1995, Lumley & Sperling 2010), genetic characters including allozymes, mitochondrial DNA, microsatellites, and genomic single nucleotide polymorphisms (SNPs) (Stock & Castrovillo 1981; Sperling & Hickey 1994, 1995; Harvey 1996; DeVerno *et al.* 1998; Lumley & Sperling 2010, 2011a, 2011b; Brunet *et al.*

2013, 2017; Blackburn *et al.* 2017; Dupuis *et al.* 2017), and particularly life-history traits (Harvey 1967; Campbell 1967; Stehr 1967; Sanders *et al*. 1977; Volney & Liebhold 1985; Shepherd *et al.* 1995; Nealis 2005).

The life cycle of spruce budworms is similar for most taxa. Males eclose from their pupae and search for mates by following plumes of pheromone emitted by females (Silk & Kuenen 1988). Females eclose, mate, and search for suitable host plants for oviposition where they lay clusters of eggs on the needles (Campbell 1953; Morris 1963). Clusters contain between 15 and 60 eggs, depending on the species, and fecundity often exceeds 150 eggs per female (Nealis 2008). First instar larvae emerge from these eggs and spin hibernacula before overwintering (Morris 1963). After moulting to the second instar, they emerge from their hibernacula and begin feeding on either year-old needles or new shoots, depending on current-year bud flush. Larvae usually feed and develop until they reach their sixth instar, then pupate and emerge as adults to begin the process anew (Morris 1963), although the number of larval instars can vary depending on location (Candau *et al.* 2019). Larvae of some species (e.g. *C. o. biennis*) undergo an obligate second diapause by spinning a second hibernaculum once they have reached their third instar (Harvey 1967, Nealis 2005). *Choristoneura o. biennis* moult while in their hibernaculum and emerge as fourth instar larvae in the following year, then continue to feed, pupate, and finally emerge as adults (Harvey 1967). Therefore, the life cycle of *C. o. biennis* lasts two years.

The taxonomic history of conifer-feeding spruce budworm species has been reviewed by Powell (1980, 1995a), with a current summary by Gilligan & Brown (2014) and Brunet *et al.* (2017), and shows continuing uncertainty about species boundaries and relationships among these species. *Tortrix fumiferana* was described by Clemens (1865), moved to *Archips* Hübner 1822 by Graham (1929), then to *Choristoneura* by Freeman (1947). The next description of a

species currently included in the complex, *Lozotaenia retiniana* Walsingham 1879, also had initial generic uncertainty before being moved by Obraztsov (1962) to *Choristoneura* after first being moved to *Archips* by Dyar (1903). Freeman (1958) moved *Archips lambertiana* (Busck 1915) into *Choristoneura* (first assigned to *Tortrix* by Busck in 1915 then moved to *Archips* by McDunnough in 1939). *Tortrix carnana* was described by Busck and Barnes 1920 but later moved to *Choristoneura*.

Several other species were recognized as being closely related to *C. fumiferana* in their first descriptions. *Choristoneura pinus* was considered to be a pine-feeding population of *C. fumiferana* until its formal species assignment by Freeman (1953). Freeman (1967) then described *Choristoneura biennis*, *Choristoneura occidentalis*, and *Choristoneura orae*. Each had previously been considered a population of *C. fumiferana* until that point. *Choristoneura occidentalis* was renamed *C. freemani* by Razowski (2008) because the specific epithet *occidentalis* was in use by an African species that Razowski reassigned to *Choristoneura* from *Cacoecia* Hübner 1825. Little evidence supported this change, and Brunet *et al*. (2017) rejected it. In the same paper, they synonymized *C. biennis* and *C. occidentalis* Freeman due to their similarity in genome-wide single nucleotide polymorphisms (SNPs). These taxa are now considered subspecies of *C. occidentalis* Freeman, i.e. *C. o. biennis* and *C. o. occidentalis*.

Temporal Isolating Mechanisms Among *Choristoneura* spp.

Smith (1953) marks the first major study of mechanisms that may reduce or prevent hybridization among members of the spruce budworm species complex. Part of his study evaluated the role of seasonal temporal isolation in maintaining sympatric populations of *C. fumiferana* and *C. pinus* in central Canada. Smith demonstrated that *C. fumiferana* emerges from

larval diapause approximately 15 days earlier than *C. pinus* when the two species are found in the same locality, thereby stopping contact between reproductive adults. Lab-reared hybrids had intermediate larval development rates relative to the parental populations, suggesting genetic control of the trait. Smith (1953) concluded that close synchrony between diapause termination and host plant development allowed these species to diverge, and that females have an "innate repugnance" for males of the opposite species, although hybrids could still be obtained in no-choice crosses. We now know this repugnance is derived from differences in male-produced sex pheromones (Roscoe, Silk, & Eveleigh 2016). In a follow-up study, Smith (1954) found occasional overlap between the flight periods of these species, but again concluded that these species do not naturally hybridize.

Temporal isolation between *C. o. occidentalis* and *C. retiniana* was evaluated in southern Oregon, where the primary hosts of these species have considerable overlap (Volney *et al.* 1983). There, these species have similar flight phenology across the season (Liebhold & Volney 1984; Volney & Liebhold 1985). The authors of these studies suggested that the species are not closely associated with their primary hosts in southern Oregon, as larvae of both species are often found feeding on both hosts (Volney *et al.* 1983) and there is little phenological difference in the timing of local bud flush of their host conifers (Volney *et al.* 1983, Liebhold & Volney 1984). They concluded that seasonal temporal isolation does not significantly affect hybridization between *C. o. occidentalis* and *C. retiniana*. Moreover, Volney & Liebhold (1985) found that lab-generated hybrids between *C. o. occidentalis* and *C. retiniana* had intermediate development rates, again suggesting genetic control of phenological traits in the spruce budworm species complex.

Powell & De Benedictus (1995) found that *C. retiniana* has major temporal overlap with two other spruce budworm species in California. In this study, *Choristoneura retiniana* and

Choristoneura carnana only had a 2- to 3-day difference in peak flight period. They observed a similar trend between *C. retiniana* and *Choristoneura lambertiana subretiniana* Obraztsov 1962. Like Volney and Liebhold (1985), they concluded that these taxa are probably not reinforced by seasonal temporal isolation.

Lumley and Sperling (2011b) found evidence of partial seasonal temporal isolation between *C. fumiferana* and *C. o. occidentalis* in Cypress Hills Provincial Park, Alberta. This was the first study of temporal isolation among spruce budworms to employ genetic markers to confirm identifications. They identified hybrids using a combination of mitochondrial and nuclear DNA and found that *C. fumiferana* had a substantially earlier flight period than *C. o. occidentalis* (Lumley & Sperling 2011b). Wild hybrids were collected throughout the *C. fumiferana* flight period, but early in the flight period of *C. o. occidentalis*. Douglas fir, the primary host of *C. o. occidentalis*, is not found in Cypress Hills (Lumley & Sperling 2011b), suggesting that larvae were feeding on an atypical spruce host or that this budworm taxon is not endemic to the area. The wings of many adult *C. o. occidentalis* did not have the degree of wing wear that may be expected in dispersing spruce budworm moths (Rhainds & Broderson 2012; Rhainds 2015), and so it is less likely that they came from an outbreaking population in the Rocky Mountains west of the collection locality (Brunet *et al.* 2013). Therefore, *C. o. occidentalis* collected in Cypress Hills may have developed locally on spruce, yet had an appreciably later flight period than *C. fumiferana*.

In addition to seasonal isolation, there is some evidence that different spruce budworm species may have separate daily flight times, although the difference is probably not enough to stop hybridization. First documented by Smith (1953), female *C. fumiferana* accept conspecific mates 1.5 to 2 hours earlier in the evening than do *C. pinus.* Sanders (1971) found that female *C.*

fumiferana "calling" (everting pheromone producing glands on the abdomen) began at sunset, whereas *C. pinus* calling began about 1.5 hours after sunset. Both species had the highest number of females calling at 22:00 hours, although the *C. fumiferana* peak was observed approximately two weeks before *C. pinus*. He predicted that the greatest number of female *C. pinus* would call about 30 minutes later than *C. fumiferana* if the species are observed on the same night (Sanders 1971). Sanders (1977) later evaluated the daily calling behaviour of other spruce budworms in a laboratory, again finding that there was considerable temporal overlap among calling females of each species. He found that >50% of female *C. o. biennis* called six hours before "sunset" (lights off) and >50% of female *C. fumiferana* called three hours before lights off, but all individuals of both species called one hour after lights off and continued to call until the experiment ended two hours later. Finally, Liebhold and Volney (1984) found that peak flight phenology between *C. o. occidentalis* and *C. retiniana* differed only slightly during a 24-hour period.

Pheromone Isolating Mechanisms Among *Choristoneura* spp.

Conifer-feeding species of *Choristoneura* can be split into two groups based on sex pheromones emitted by adult females. *Choristoneura fumiferana*, *C. o. occidentalis*, *C. o. biennis*, and *C. carnana* each use *E*/*Z*11 tetradecenal, although the exact ratio of *E* to *Z* isomers is not known for *C. o. biennis* or *C. carnana* (Silk & Eveleigh 2016). *Choristoneura pinus*, *C. orae*, *C. retiniana*, and *C. lambertiana* each use *E*/*Z*11 tetradecenyl acetate and *E*/Z11 tetradecen-1-ol in a ratio of approximately 9:1 (Silk & Eveleigh 2016). Despite the differences in chemical ecology between these two groups, cross-group hybrids can still be obtained in laboratory (Harvey 1967, 1997; Volney *et al.* 1984; Powell 1995b) and natural settings (Smith 1953, Powell 1995b; DeVerno *et al.* 1998; Lumley & Sperling 2011a, 2011b; Brunet *et al.* 2017), and males of

either pheromone group are attracted to both *E*/*Z*11 tetradecenyl acetate with *E*/*Z*11 tetradecen-1-ol and *E*/*Z*11 tetradecenal (e.g. Liebhold *et al*. 1984; Powell 1995b).

E/*Z*11 tetradecenyl acetate is a precursor to *E/Z*11 tetradecenal (Morse & Meighen 1984, 1986; Silk & Kuenen 1988), so selection may have led to a distinct sex pheromone from the ancestral *E*/*Z*11 tetradecenyl acetate. This would have allowed diverging aldehyde users to identify conspecifics in the face of gene flow from acetate/OH users. However, *E/Z*11 tetradecenals or similar molecules also seem to be used by other tortricids for mate-finding, as demonstrated by their frequent bycatch in *C. fumiferana* pheromone-baited traps (Weatherston *et al.* 1978; Brewer, Krampl, & Skuhravý 1985; Sanders 1993; Ostrauskas, Ivinskis, & Būda 2010). It may be that subtle differences in the production of tortricid sex pheromones have been evolved and lost repeatedly. Notably, *C. orae* uses *E*/*Z*11 tetradecenyl acetate and *E*11 tetradecen-1-ol (Silk & Eveleigh 2016) but is closely related to *E/Z*11 tetradecenal users (Dupuis *et al.* 2017). It also has a two-year life cycle like that of *C. o. biennis* (Freeman 1967) and is mostly allopatric with other conifer-feeding *Choristoneura* (Lumley & Sperling 2011a). This suggests that *C. orae* either shares ancestry with or is derived from the *E/Z*11 tetradecenal users but has reverted to acetate/OH use after long-term allopatry on the western coast of Canada. In contrast, *C. lambertiana* also uses *E*/*Z*11 tetradecenyl acetate and *E*/*Z*11 tetradecen-1-ol (Silk & Eveleigh 2016) and is closely related to *E/Z*11 tetradecenal users (Dupuis *et al.* 2017) but is largely sympatric with other spruce budworms (Lumley & Sperling 2011a).

Population density also affects spruce budworm mating. Male *C. fumiferana* have greater mating success when many conspecific females are present (Kipp, Lonergan, & Bell 1995; Régnière, Delisle, Pureswaran, & Trudel 2013). However, mating success is reduced at very high population density, possibly driving adult dispersal (Régnière *et al.* 2013). Thus, hybridization

between spruce budworms appears to occur most frequently at intermediate population densities, with high population density presumably causing competition between any one source of female sex pheromone and impeding mating. The forest tent caterpillar (*Malacosoma disstria* Hübner 1820), another lepidopteran with cyclical outbreaks, also has its greatest mating success at intermediate population densities (Evenden, Mori, Sjostrom, & Roland 2015), suggesting this phenomenon may be common among Lepidoptera with cyclical outbreaks. It is also possible that hybridization frequency among spruce budworm species varies across their ranges due to subtle differences in pheromone blend, as has been found in other tortricids (e.g. Emelianov, Drès, Baltensweiler, & Mallet 2001; Bengtsson *et al.* 2014).

Silk and Eveleigh (2016) have suggested that the behaviour and/or pheromones of males, after being drawn to a calling female, may be a further basis for interactions that could be important in congeneric (and conspecific) mating events. For example, Roscoe, Silk, & Eveleigh (2016) found that male *C. fumiferana* had lower mating success if male sex pheromone producing structures (hair pencils) were removed, demonstrating the importance of close-proximity male-produced chemicals.

Mechanical Isolating Mechanisms Among *Choristoneura* spp.

There is little evidence to suggest that mechanical barriers reduce hybridization among conifer-feeding spruce budworms. Dang (1985) developed a key for conifer-feeding *Choristoneura* that used spicules on male genitalia as a diagnostic character, and followed this with a detailed study of spicules in the genus (Dang 1992). However, Gray *et al*. (1995) and Shepherd *et al.* (1995) found that spicules alone were ineffective for species identification as each species varies in its spicule frequency. For example, about 20% of *C. fumiferana* males

have no spicules on their genitalia, but others can have more than 300 (Gray *et al.* 1995). In contrast, fewer than 3% of *C. o. biennis* and *C. o. occidentalis* males have spicules, although there is overlap in spicule number between *C. fumiferana* and these subspecies. These findings suggest that spiculate *versus* non-spiculate genitalia do not stop hybridization between *C. fumiferana* and subspecies of *C. occidentalis*, even though *C. o. biennis* and *C. o. occidentalis* usually have fewer spicules than *C. fumiferana*.

Larval Host-Associated Isolating Mechanisms Among *Choristoneura* spp.

Host plant association may affect hybridization in localities with multiple host species. *Choristoneura fumiferana* and *C. o. occidentalis* larval development and flight phenology is related to flushing of primary host buds (Volney & Cerezke 1992; Lawrence *et al.* 1997; Nealis 2012; Fuentealba *et al.* 2017), suggesting that temporal association with these hosts contributes to isolation between spruce budworm species. However, many spruce budworm species feed on non-primary host conifers (Furniss & Carolin 1977; Harvey 1985; Brown, Robinson, & Powell 2008). It is possible that F1 hybrids have reduced feeding and/or ovipositional preference for certain hosts, as has been observed in *C. fumiferana* on resistant *versus* non-resistant cohorts of white spruce (Daoust *et al.* 2010; Mader, Daoust, Cardinal-Aucoin, Bauce, & Despland 2012). Additionally, primary host plant association is correlated with the geographical range of each spruce budworm species (Stehr 1967; Shepherd *et al.* 1995), which must contribute to broadly reducing natural hybridization between species, although this aspect of host association would not determine hybridization in sympatric populations.

Thesis Objectives

There are many potential barriers to hybridization among the members of the spruce budworm species complex. In this thesis, I focussed on the role of both seasonal and between-year temporal isolation between *C. fumiferana* and *C. o. biennis*. Most of my research documents the phenology and distribution of adults across a region of Alberta where previous research (Brunet *et al.* 2017; Blackburn *et al.* 2017) has shown some contact between these two taxa. However, these previous studies used sampling that was too widely spaced, both temporally and geographically, to provide a clear sense of the temporal interaction between the taxa. I chose six localities in west-central Alberta where both taxa were likely to co-exist and collected adults over two years to determine the level of overlap between their flight periods. I identified 261 of the collected *Choristoneura* using genome-wide SNPs, evaluated their flight phenology, estimated the rate of hybridization between these taxa, and assessed the correspondence of their flight period with degree-day accumulation. Some *C. o. occidentalis* individuals were also collected and identified, and so I performed a preliminary analysis to identify potentially adaptive SNPs between the subspecies of *C. occidentalis*. This was not done between *C. fumiferana* and *C. o. biennis* as they differ at too many SNPs to plausibly link these to adaptive species differences. I also evaluated whether the potentially adaptive SNPs within *C. occidentalis* were in close chromosomal linkage in the genome. The results of this study comprise Chapter 2.

I also attempted two other experiments in addition to those described in Chapter 2: one to compare field attraction to different sex pheromones between *C. fumiferana* and *C. o. biennis*, and another to determine whether the larvae of these two taxa were associated with different primary hosts where they overlap in the field. For the sex pheromone study, I baited green Unitraps with either synthetic *C. fumiferana* sex pheromone (95:5 *E*/*Z*11 tetradecenal) or

putative synthetic *C. o. occidentalis* sex pheromone (92:8 *E*/*Z*11 tetradecenal) (Silk & Eveleigh 2016) and deployed both trap types in the 2018 collection localities. However, I did not collect any *Choristoneura* in the traps with pheromone that was expected to be optimized for *C. o. occidentalis*. For the larval performance study, I used a standard beating sheet technique to collect larvae from host branches (Chapter 2 for methods). Only four larvae were collected, and sequencing was successful for only two of three attempted. Further discussion of these experiments can be found in Chapter 3.

Overall, my thesis aimed to determine the extent of seasonal temporal isolation between *C. fumiferana* and subspecies of *C. occidentalis* in a region of substantial potential contact. I also estimated the natural frequency of hybridization of these species to provide further insight into the mechanism by which they species maintain their genomic integrity despite their documented ability to hybridize in laboratory studies.

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Chapter 2

Temporal isolation and genetic divergence between spruce budworm moths (*Choristoneura fumiferana* and *C. occidentalis*) in west-central Alberta and British Columbia

Introduction

One of the most obvious contributors to ecological speciation is temporal isolation, a mechanism that separates reproductive times between sympatric populations of a species (Hendry & Day 2005; Nosil 2012). Asynchronous reproductive periodicity of two populations can allow adaptive or neutral divergence over successive generations (Rundle & Nosil 2005), leading to maintenance of their genomic integrity as species (Sperling 2003). Phytophagous insects present convenient systems for studying such temporal isolation, as adaptation to host plants with divergent phenology can lead to gene-flow reduction between host-plant cohorts (Funk, Filchak, & Feder 2002; Matsubayashi, Ohshima, & Nosil 2010; Powell *et al.* 2013; Forbes *et al.* 2017). Gene-flow barriers can also arise independently of host plants, where allochronic differences alone initiate divergence between sympatric insect cohorts (Taylor & Friesen 2017). Such "allochronic speciation" (Alexander & Bigelow 1960) may occur at multiple timescales, with lepidopteran taxa showing temporal isolation by time of day (e.g. Ueno, Furukawa, & Tsuchida 2006; Devries, Austin, & Martin 2008), seasons within a year (e.g. Yamamoto & Sota 2009; Bell, Hamm, Shapiro, & Nice 2017), or between multiple years (Gradish, Keyghobadi, & Otis 2015).

The spruce budworm species complex contains eight or nine conifer-feeding species in the genus *Choristoneura* Lederer 1859 (Lumley & Sperling 2011a; Brunet *et al.* 2017). *Choristoneura fumiferana* (Clemens 1865) ranges across the boreal forest of North America

(Lumley & Sperling 2011a), overlapping with *Choristoneura occidentalis biennis* Freeman 1967 on the eastern slopes of the Canadian Rocky Mountains and *Choristoneura occidentalis occidentalis* Freeman 1967 in south-western Alberta (Lumley & Sperling 2011a). These three taxa exhibit very similar morphology (Freeman 1967; Harvey & Stehr 1967; Dang 1992; Gray, Shepherd, & Harvey 1995; Lumley & Sperling 2010; R. L. K. French, personal communication), sex pheromones (Sanders, Daterman, & Ennis 1977, Silk & Kuenen 1988; Silk & Eveleigh 2016), and life-history traits (Campbell 1967; Volney & Fleming 2007; Nealis 2005; Brunet, Hundsdoerfer, & Sperling 2013). There is also some overlap in their larval hosts (Furniss & Carolin 1977; Harvey 1985; Brown, Robinson, & Powell 2008). Therefore, genetic characters have become the most reliable means for their identification (Sperling & Hickey 1994, 1995; Harvey 1996; Lumley & Sperling 2010, 2011a; Blackburn *et al.* 2017; Brunet *et al.* 2017; R. L. K. French, personal communication). Our use of taxonomic names within *Choristoneura* follows Brunet *et al.* (2017), who considered *C. o. biennis* to be a subspecies of *Choristoneura occidentalis* based on similarity in genome-wide single nucleotide polymorphisms (SNPs). Hereafter, we refer to these taxa as *fum*, *bie*, and *occ*.

There is evidence of seasonal temporal isolation between multiple species pairs in the spruce budworm species complex. Smith (1954) found that sympatric *fum* and *Choristoneura pinus* Freeman 1953 differ in peak adult flight phenology by approximately two weeks. Lumley and Sperling (2011b) found that *fum* and *occ* have differing adult flight phenology with some hybridization in an isolated forest island in southeastern Alberta, Canada. In contrast, Volney, Waters, Akers, and Liebhold (1983) found that populations of *occ* and *Choristoneura retiniana* (Walsingham 1879) had similar adult flight periods within one season, with occasional hybridization. Powell and De Benedictus (1995a) also found similar flight periods between

populations of *C. retiniana* and *Choristoneura carnana* (Barnes & Busck 1920) as well as *C. retiniana* and *Choristoneura lambertiana subretiniana* Obraztsov 1962.

Voltinism may contribute to temporal isolation among spruce budworm species by causing between-year differences in emergence. Adult females of conifer-feeding *Choristoneura* oviposit on host plant foliage (Campbell 1953), then neonates spin hibernacula and enter diapause before overwintering (Morris 1963; Harvey 1967). In the following year, larvae emerge and begin feeding on host foliage. Larvae of *fum* soon pupate to complete a single-year life cycle (Morris 1963). Conversely, larvae of *bie* undergo an obligate second diapause and overwinter again before completing a two-year life cycle (Harvey 1967, Nealis 2005). This two-year life cycle leads to oscillations wherein adults fly in larger numbers every second year, separated by a year of few adults (Zhang & Alfaro 2002; Nealis & Turnquist 2003). This suggests that between-year temporal isolation may also reduce gene flow between *fum* and *bie*.

Most spruce budworm species readily hybridize with each other in laboratory settings, but naturally-occurring hybrids appear to be uncommon (Smith 1953; Campbell 1967; Harvey 1967, 1985, 1997; Sanders *et al.* 1977; Volney, Liebhold, & Waters 1984; Volney & Liebhold 1985; Powell & De Benedictus 1995a, 1995b, Nealis 2005). Therefore, it is likely that prezygotic mechanisms reduce hybridization between congenerics. Here, we assess the role of temporal isolation in maintaining the genomic integrity of taxa in the spruce budworm species complex. We collected specimens from areas with documented overlap between *fum* and *bie* during two successive years to compare their flight phenology. We used genome-wide SNPs to estimate the frequency of hybridization between *fum* and *bie*, and to distinguish *bie* from *occ.* We also evaluated the correlation between flight phenology and within-locality degree-day accumulation. Finally, we examined the chromosomal position of markers contributing to the genomic

differences between *bie* and *occ*. We found that there were appreciable differences between the peak flight periods of *fum* and *bie*, both within a flight season and between years. These differences may help to reduce gene flow but contact leading to hybridization is still possible through substantial overlap in flight period. We also found that the range of *C. o. biennis* extends >100 km farther east than previously recorded. Our study is the first fine-scale evaluation of hybridization and temporal isolation between *C. fumiferana* and both subspecies of *C. occidentalis.*

Methods

Sampling

Spruce budworm species were sampled at 10-day intervals during their potential flight seasons in 2017 and 2018 at each of 10 localities in western Canada (Figure 2-1; Table 2-1; Table 2-2). Six localities (3–8, Figure 2-1) were in the known overlap zone of the ranges of *fum* and *bie* (Brunet *et al.* 2017). Individuals from locality 9 were expected to include only *fum*, as it is geographically distant from the documented range of *bie* (Lumley & Sperling 2011). Likewise, we collected *bie* individuals from localities 1, 2, and 10, where it is geographically isolated from *fum* by the Rocky Mountains (Lumley & Sperling 2011; Brunet *et al.* 2017). Localities 1, 2, and 10 were chosen based on aerial surveys depicting severe *bie* defoliation in 2015 and 2016 (Westfall & Ebata 2016, 2017), two years prior to our collections.

Adult sampling occurred during 13 June – 25 August 2017 and 1 June – 3 September 2018 (Table 2-1, Table 2-2). Four green Unitraps (Contech, Delta, British Columbia, Canada) were hung at eye level from mature host plants at each locality, with at least 40 metres between each trap to reduce interference (Allen *et al*. 1986; Sanders 1996). Three Unitraps were baited

with rubber septum lures containing 100 μg of synthetic 95:5 *E*,*Z*-11-tetradecanal (Sylvar Technologies, Fredericton, New Brunswick, Canada), the dominant component of the *fum* sex pheromone (Silk & Kuenen 1988). The fourth trap did not contain a lure, thereby acting as a control. The 95:5 *E,Z* blend developed for *fum* effectively attracts other members of the genus, including both subspecies of *C. occidentalis* (Sanders *et al.* 1977; e.g. Lumley and Sperling 2011a, 2011b; Brunet *et al.* 2017), although the optimum *E*/*Z* ratio for *bie* is unknown (Sanders 1971; Sanders, Daterman, Shepherd, & Cerezke 1974). Each trap also contained one strip of 10% dichlorvos as a killing agent (Hercon Environmental, Emigsville, Pennsylvania, USA). Trap catch was collected approximately every 10 days and placed into -20°C freezers at the University of Alberta upon return to Edmonton.

After the 2017 collection season, it was not clear whether we had sampled *bie* during its peak emergence year. Examination of aerial surveys from the previous two years did not resolve whether even- or odd-year defoliation was dominant near our *bie*-only localities (Westfall & Ebata 2016, 2017). Therefore, we continued to survey spruce budworm moths throughout the 2018 collection season. We also added locality 10 to the survey in 2018, based on clearer prior records of even-year severe defoliation by larval *bie* in the area (Westfall & Ebata 2016).

Larval collections were undertaken at localities 1–9 during 24 May – 17 June 2017 (Table 2-1), using a protocol modified from Sweeney, McLean, and Shepherd (1990). We used a beating sheet of white fabric stretched over two wooden dowels and measuring 54 by 54 cm. The sheet was held below the terminal 45 cm of two mature host branches while shaking the branch ten times to dislodge any larvae. Larvae were placed in vials with 95% ethanol, then transferred to a -20°C freezer at the University of Alberta. This process was repeated for branches on ten white spruce, *Picea glauca* (Moench) Voss, and ten Douglas fir, *Pseudotsuga menziesii* (Mirb.)

Franco, the respective primary hosts of *fum* and *occ* (Shepherd, Gray, & Harvey 1995), at localities 1 and 2 on each sampling visit, depending on host availability. Only white spruce was sampled at localities 3-9, as Douglas fir was not present. Each tree was sampled only once, and the number of current-year buds was recorded on each sampled branch to quantify sampling effort. The primary hosts of *bie*, subalpine fir, *Abies lasiocarpa* (Hook.) Nuttall, and Engelmann spruce, *Picea engelmannii* Parry ex. Engelmann (Shepherd *et al.* 1995) were not identified at any of the localities.

DNA Extraction and ddRADseq

Adult specimens were arbitrarily subsampled for genotyping from any collection events with multiple adults. To maximize coverage of flight period, we also selected singletons that were trapped earliest or latest in each collection season. Most collection periods were represented by at least three individuals, if specimens were available. Up to 10 individuals were genotyped from collection periods with more numerous samples. When we could not genotype all individuals from one collection period, we selected both fresh and worn moths to cover the breadth of variability present. Individuals that were wet upon collection were not selected. Figure 2-2 contains a flowchart of subsequent methods.

We extracted DNA from the subsampled individuals which was then sequenced in the Molecular Biology Servicing Unit (MBSU) at the University of Alberta. Head and thoracic tissue was extracted from larvae, while thoracic or anterior abdominal tissue was used for adults. The remaining wings, head, and abdomen of each adult were retained for future study. DNA was extracted using DNeasy Blood & Tissue DNA Kits (QIAGEN, Hilden, Germany) with bovine pancreatic ribonuclease A (QIAGEN). DNA of each specimen was ethanol precipitated and

resuspended in millipore water for further concentration and purification, then kept in -20°C freezers until library preparation. *PstI-MspI* restriction enzyme ddRADseq libraries were prepared using the protocol of Peterson, Weber, Kay, Fisher, and Hoekstra (2012) in the MBSU. We did not use the size selection step outlined in Peterson *et al.* (2012), as this complexity reduction is not required to obtain sufficient genotypic data for species identification (e.g. Blackburn *et al.* 2017; Brunet *et al.* 2017). Single-end, 75 base-pair (bp) sequencing was performed on an Illumina NextSeq 500 platform. The data generated for this study were sequenced on three separate NextSeq runs, and four individuals were sequenced twice to evaluate whether there were differences between runs.

DNA Data Processing

We performed initial bioinformatic processing of Illumina-generated FASTA files on supercomputing clusters of WestGrid (Compute Canada, Kelowna, British Columbia, Canada). The data were demultiplexed using the *process_radtags* program in Stacks version 2.3d (Catchen, Hohenlohe, Bassham, Amores, & Cresko 2013). We discarded any reads with a Phred score <20 over 15% of the read length, and any reads that failed the Illumina purity filter. The retained reads were trimmed from 75 bp to 67 bp after removing the 8 bp index sequence. Some sequencing error was identified in the *PstI* site, so another 5 bp were removed from the 5' end of the reads with Cutadapt version 1.18 (Martin 2011). The trimmed read length was 62 bp.

We aligned the trimmed reads to 30,517 scaffolds of *fum* draft genome bw6 (Cusson *et al.* unpublished) to increase our read depth and remove non-target reads, then filtered low quality SNPs. Alignment was performed using the Burrows-Wheeler Aligner version 0.7.17-r1188 (*bwtsw* algorithm, Li & Derbin 2009). We converted SAM files output by the aligner to BAM

files that we then sorted and ran through the Stacks pipeline using the *ref_map* program. This runs *gstacks* to assemble aligned loci before calling SNPs, then runs *populations* to generate population genetic statistics (Catchen *et al.* 2013). We used a population map composed of a single population containing all individuals. SNPs present in ≥80% of the individuals were retained, as were those with a minor allele frequency ≥10%. Stacks-filtered SNPs were further filtered in VCFtools version 0.1.14 (Danecek *et al.* 2011). We retained only those that were biallelic, had a minor allele frequency of ≥10%, and had ≤5% missing data across individuals. We also selected SNPs that were no closer than 10k bp to one another, minimizing the effect of physical linkage on a single scaffold of the draft genome.

Taxonomic Identifications

Spruce budworm species identities were first determined with clustering in principal component axes. Processed VCF files were loaded into RStudio version 1.1.463 (RStudio Team 2016) using version 1.8.0 of the vcfR package (Knaus & Grünwald 2017). Each VCF file was converted to a genlight object and analyzed by principal components analysis (PCA) using version 2.1.1 of the adegenet package (Jombart & Ahmed 2011). PCA clusters were evaluated after visualisation using ggplot2 (Wickham 2017).

Bayesian clustering analysis in *structure* version 2.3.4 was also used for species identification, as well as finding introgression between taxa (Pritchard, Stephens, & Donnelly 2000; Falush, Stephens, & Pritchard 2003). We used the same, filtered VCF files in these analyses that we used for the PCAs. The VCFs were converted to genepop files using PGDSpider version 2.1.1.5 (Lischer & Excoffier 2012), then to STR files using in-house Bash code. We ran ten iterations of $k = 1–10$ with a burn-in period of 100,000 followed by 1,000,000

Markov chain Monte Carlo generations. This was done using *locpriors* corresponding to the 10 collection localities, which better resolves weak population structure (Porras-Hurtado *et al.* 2013). We used StructureSelector (Li & Liu 2018) to determine the number of genetic clusters with the highest support, with LnP(*k*) (Pritchard *et al.* 2000) and Δ*k* (Evanno, Regnaut, & Goudet 2005). CLUMPAK version 1.1 (Kopelman *et al.* 2015) was used to determine the average run between the 10 replicates of each *k* and to create matrices of membership coefficients (Jakobsson & Rosenberg 2007). The membership coefficients were used to create *structure* plots.

From the first *structure* run, individuals were identified as either *fum* or *C. occidentalis* (*bie* + *occ*) if they had >90% membership to either genetic cluster, otherwise they were considered admixed. We then used hierarchical testing to find substructure within the two major clusters, i.e., we re-ran *structure* separately on each of the genetic clusters from the run with the highest supported value of *k*. Putative F1 hybrids, those with approximately 50% membership to both the *fum* and *bie* + *occ* clusters, were not included in subsequent *structure* analyses as they were not part of either genetic cluster. We did include two admixed individuals that had between 65% and 90% membership in a major cluster. Within the *C. occidentalis* cluster, individuals were considered *bie* if they had >50% membership to cluster one and *occ* if <50% membership. Finally, we ran *structure* again on only those individuals that had >90% assignment to the *bie* cluster to detect potential genetic structuring between the odd-year and even-year cohorts. The frequency of hybrids between *fum* and *bie* + *occ* was estimated by comparing the frequency of F1 hybrid individuals to non-F1 hybrids.

Evaluation of Temporal Isolation

Genotyped specimens were used to evaluate adult flight phenology between *fum* and *bie*, as well as between *bie* and *occ*. To explain differences in flight phenology between these taxa, we modelled within-locality thermal accumulation using degree-day calculations (Zalom, Goodell, Wilson, Barnett, & Bentley 1983). Régnière (1990) demonstrated that photoperiod has minimal effect on post-diapause emergence of *fum*, so we only evaluated degree-days. We recorded locality-specific degree-day accumulation using HOBO Pendant Temperature/Light Data Loggers (Onset Computer Corporation, Bourne, Massachusetts, USA) during the 2018 collection season. These data were used to calibrate temperature data recorded by weather stations near each locality. We hung one HOBO Logger at eye level at each locality. Temperature data were retrieved from the loggers using HOBOware version 3.7.12 (Onset Computer Corp.). We downloaded local temperature data from weather stations near each locality (Table 2-3), choosing the closest station to each. When no stations were within 15 km of a locality, we compared multiple closest stations (Table 2-3). Data were downloaded from all selected stations and in-house Bash code was used to isolate the highest and lowest daily temperatures between trap set-up and the trap removal from each locality, each year (Table 2-1, Table 2-2). Daily high and low temperatures were also isolated from the logger data for each full calendar day that they were deployed. We then compared each of the daily high temperatures recorded by the loggers to those of the nearest weather station (Table 2-4). Regression models were used to visualize locality-specific trends between logger and station data, and Wilcoxon-Mann-Whitney tests were used to evaluate differences between them. We used the difference between the medians of these distributions as a station-to-locality correction factor; the difference was applied to all daily high temperatures recorded by the weather station between 1

January and the day traps were removed from each locality, each year. A correction factor was also determined and applied to the daily low temperatures. Correction factors were applied irrespective of Wilcoxon-Mann-Whitney test significance.

Degree-day accumulation was calculated using the single triangulation method (Lindsey & Newman 1956; Zalom *et al.* 1983). This method is more accurate than alternatives when temperatures are far below the lower developmental threshold of an insect (Roltsch, Zalom, Strawn, Strand, & Pitcairn 1999). We used 5.5°C as the lower development threshold for both *fum* and *bie.* The threshold for *bie* has not been determined, but 5.5°C has previously been used for comparing development between *fum*, *bie*, and *occ* (Shepherd 1961; Kemp, Dennis, & Beckwith 1986). We did not use an upper developmental threshold. Thirty-eight°C, the upper developmental threshold used for *fum* larvae in the Régnière phenology model (Régnière, Saint-Amant, & Duval 2012 and references therein), was only exceeded during one hour-long period at locality 8. The upper developmental threshold temperature of *bie* is unknown. Finally, we plotted the number of genotyped individuals collected on each day against the number of within-locality accumulated degree-days (i.e. calendar time *versus* physiological time), then evaluated the correspondence of these variables. This allowed better comparison of phenology between disparate localities. We projected 80% confidence ellipses to evaluate overall flight period synchrony.

Genomic Location of SNPs

To identify the genomic regions associated with divergence between *bie* and *occ*, we performed BLASTX searches using flanking sequences around high-loading SNPs of PC1 of the PCA of *occ* + *bie* specimens. SNPs were considered high-loading if they had a contribution

≥0.005 on the loading plot. We used the draft genome to retrieve 10 kb on either side of each SNP, or all bases between the SNP position and the end of the scaffold if it ended before 10 kb was met. We then used BLASTX version 2.9.0+ on the non-redundant sequence database of NCBI to find protein matches for these regions (Altschul *et al.* 1997) and inferred the function of the top sequence match using gene ontology terms and associated publications. Additionally, we determined the location of these SNPs on the *fum* linkage map (Picq *et al.* 2018) to evaluate whether major genetic differences between *bie* and *occ* are restricted to linkage groups.

Results

Collections

A total of 8,285 adult male individuals were collected using green Unitraps baited with synthetic *fum* sex pheromone (Figure 2-1). Of these, 1,011 were collected during the 2017 collection season, and 7,242 during the 2018 collection season. Collection totals are listed by locality in Appendix 2-1. In 2017, peaks in flight period were observed in early and mid-July across all nine collected localities. In 2018, peaks in flight period were observed between late June and early July at most localities, and a second peak was observed later in the season at some localities. Most notably, the three localities where *bie* is geographically isolated from *fum* exhibited two flight period peaks in 2018 (Figure 2-1, localities 1, 2, and 10).

Four larvae were collected using the beating sheet protocol (Table 2-5). A total of 310 white spruce branches were sampled on 155 individual trees, amounting to 35.9 ± 15.2 new-growth buds sampled per branch. Only 50 Douglas fir branches were sampled on 25 individual trees, amounting to 26.2 ± 8.9 new-growth buds sampled per branch. We did not find Engelmann spruce at any of the localities, though some white spruce may have been hybrids between the two

species, or even improperly identified Engelmann spruce (Strong & Hills 2006; Haselhorst, Parchman, & Buerkle 2019). However, both white and Engelmann spruce are suitable hosts for *fum* as well as *bie* (Furniss & Carolin 1977; Brown *et al.* 2008).

ddRAD SNPs

After DNA extraction and sequencing, we filtered raw reads from 267 individuals. All 267 were run through Stacks; however, five had >30% missing data and were removed before a subsequent run. *gstacks* retained 319,411 loci, comprised of 113 million total reads (mean of approximately 432,500 reads aligned per individual). Mean read depth was 16.6x across all loci. These loci were further filtered in *populations* to obtain 13,243 SNPs. Final filtering in VCFtools retained 2,975 SNPs. We removed one additional individual that now had >30% missing data in the second run, bringing our total number of individuals to 261. We found no evidence of run effects using principal component analysis using a minor allele frequency of 10%.

Identity of Taxa and Hybrids

We identified unknown individuals using principal components analysis. Records of genotyped specimens are listed in Appendix 2-2. The 261 individuals had 2.69% mean missing data across 2,975 binary SNPs. The individuals clustered into three groups along principal components axis 1 (PC1) (Figure 2-3), which explained 52.91% of the variance. Of these, 102 individuals were identified as *fum* and 152 as *C. occidentalis* (*sensu* Brunet *et al.* 2017). Five individuals were identified as putative F1 hybrids between the species due to intermediate clustering on PC1 and approximate 50% membership to either *structure* cluster. The final two individuals were considered to be non-F1 admixed individuals due to their *structure* membership

(Figure 2-3). We found the greatest support for two genetic clusters among the 261 individuals (likelihood = -513,572.4; Δk = 2–9 equals 99332.5, 16.3, 0.7, 0.9, 15.5, 0.8, 1.4, 0.3).

The dataset was re-filtered to isolate the *C. occidentalis* individuals and determine whether substructure exists within them. We removed hybrid F1s from this analysis. PCA found that *Choristoneura occidentalis* was split into two discrete clusters (Figure 2-4). The 142 individuals that were assigned to the largest cluster were identified as *bie* because there were many more collected in 2018 than in 2017, as would be expected by its two-year life cycle (Nealis 2005). The remaining 11 individuals from cluster one were identified as *occ*, and they were primarily from more southerly localities in British Columbia, within plausible dispersal distance of the known range of *C. o. occidentalis*. Genotyping was reinforced by *structure*, as the greatest support was found for two genetic clusters (likelihood = -215,336.5; Δk = 2–9 equals 240.4, 0.2, 1.8, 4.0, 1.0, 1.0, 0.5, 0.03). Individuals found in each PCA cluster corresponded to clusters identified in *structure*, although one individual had 60% membership to cluster two but was still considered to be *occ* due to its location in the PCA (Figure 2-4).

PCA clustering found no apparent substructure within each of *bie* or *fum* (Figure 2-5; Figure 2-6). This was further supported by incongruence between the likelihood and Δk methods, in addition to low support for any one Δk value (*bie* - Δk = 2–9 equals 1.6, 0.2, 1.1, 2.3, 5.0, 0.3, 0.6, 0.5; *fum* - Δk = 2–9 equals 0.6, 1.6, 0.4, 1.8, 1.2, 1.3, 0.6, 0.3; *structure* barplots not shown).

We determined the rate of hybridization between *fum* and *C. occidentalis* to be 2.9% in the localities where their ranges were known to overlap (Table 2-6). Lumley and Sperling (2011b) and Brunet *et al.* (2013) found hybridization rates of 7.3% and 7.0% in localities where species overlap in southern Alberta, as determined by *fum* and *occ* microsatellite and

mitochondrial marker mismatch, while Brunet *et al.* (2017) found a hybridization rate of 1.1% for *fum* and *C. occidentalis* across their overlapping range from southern to central Alberta.

Temporal Isolation Within and Between Species

We found that flight periods were different between genotyped *fum* and *bie*, but isolation was not complete at any of the overlap localities (Figure 2-7; Figure 2-8, localities 3–9). Overall, the median date of *fum* flight was significantly earlier in the season than that of *bie* at these localities ($U = 5253$, $p < 0.001$, $r = 0.67$; Figure 2-9), with a peak flight difference in 2018 of approximately four weeks. Adult *fum* were genotyped in similar numbers for each year (52.4% of *fum* were collected in 2017, n individuals = 54 *versus* 47.6% in 2018, n individuals = 49). Adult *bie* were genotyped at much higher frequency in 2018 than in 2017 across all overlap localities (Figure 2-8). Our genotyping for overlap localities 3–8 only revealed *bie* at localities 3 and 5 in 2018; no *fum* were genotyped from those localities during that collection season. We also collected four *bie* from locality 9, which is >100 km outside its known range (Lumley & Sperling 2011a). Larval genotyping confirmed that *bie* were endemic at locality 1 and that *fum* were endemic at locality 8 (Figure 2-3). Hybrid individuals were often collected during peak *fum* flight period (Figure 2-7). Corrected degree-day accumulation for genotyped *fum* was significantly different than that of *bie* ($U = 1701$, $p < 0.001$, $r = 0.41$; Figure 2-9).

We collected two genetically distinct cohorts of *C. occidentalis* in localities 1 and 2 (Figure 2-8). No *occ* were identified in the collected material from locality 10, and only one *occ* was identified among the overlap localities (Figure 2-8, locality 3). Neither PCA clustering nor *structure* analysis found any significant genetic differences between *bie* collected in 2017 *versus*

2018 (Figure 2-5, *structure* barplot not shown). Additionally, adult *C. occidentalis* had two substantial peaks in flight period at locality 1 in 2018 (Figure 2-1).

Range Extension of C. occidentalis biennis

We identified four *bie* individuals collected at locality 9, extending its known range >100 km east (Figure 2-1; Figure 2-8). Lumley and Sperling (2011a) previously documented that putative *bie* adults can be trapped at Edson, Alberta. They also collected adults to the east of Edson that had mitochondrial haplotypes or microsatellite profiles that may indicate *bie* using *E*/*Z*11 tetradecenal lures, but also to *C. pinus* acetate lures to which *bie* are not normally attracted (Silk & Eveleigh 2016). Thus, their specimens could have been *C. pinus*, *C. lambertiana*, or hybrids that also occur in this region (Lumley & Sperling 2011a). Our *bie* were collected at least 100 km further east than previous unambiguous *bie* records.

Identity and Location of High-loading SNPs

We used BLASTX to identify six SNPs that made the largest contribution to differences between *bie* and *occ* (Figure 2-10; Table 2-7). Two of these six SNPs were associated with regions encoding heat shock proteins. One of the heat shock proteins had already been identified as being under selection between *bie* and *occ* by Blackburn *et al.* (2017), in a study that was based entirely on different specimens from those used here but from the same geographic area. We also examined 18 other high-loading SNPs (Table 2-8). In total, three of the 24 had matches on the *fum* linkage map (Picq *et al.* 2018). Two high-loading SNPs were on linkage group eight, and one was on linkage group six.

Discussion

Confirmation of Hybridization Between fum *and* C. occidentalis

Our data showed the hybridization rate between *fum* and subspecies of *C. occidentalis* to be 2.9% across their overlap localities (Table 2-6, Figure 2-3). This can be compared to previous studies that found over 7% of *fum* and *occ* have nuclear and mitochondrial markers that are assigned to the opposite species (Lumley & Sperling 2011b; Brunet *et al.* 2013). Brunet *et al.* (2017) used genome-wide SNPs to estimate a hybridization rate of 1.1% across the overlapping range of *fum* and *C. occidentalis*, but their sampling was more spatially and temporally coarse-grained as they focussed on exploring the genetic relationships among taxa. These previous studies provide good evidence of natural hybridization and/or incomplete lineage sorting between these taxa, but they did not directly quantify hybridization rate. Our work, conducted across two full seasons, provides a more fine-grained examination of the hybrid zone between *fum* and subspecies of *C. occidentalis* and should comprise a more appropriate estimate of their rate of hybridization over a full season.

Temporal Isolation Within and Between Species

We found that temporal isolation between *fum* and *bie* is incomplete where their ranges overlap (Figure 2-7), providing an opportunity for them to contact one another and potentially hybridize. In 2017, late-flying *fum* were collected at the same time as early-flying *bie*, but not in appreciable numbers. In 2018, *fum* were collected at the same time as *bie* on six of seven occasions. This suggests that much of contact between taxa occurs every second year when *bie* is flying is greater numbers. Given the extent of this contact, we would have expected to collect F1 hybrids and backcrosses in greater proportion. Figure 2-3 demonstrates that introgression is

possible between these taxa, but the number of hybrid individuals is constrained. Four F1 hybrids and one other admixed individual were collected during peak *fum* flight, one F1 hybrid during peak *bie* flight, and one admixed individual during late *bie* flight.

Volney and Cerezke (1992) noted that post-diapause *fum* emergence is in tight synchrony with white spruce bud flush, and Lawrence, Mattson, and Haack (1997) found high larval mortality if *fum* emergence occurs after host flushing. This is also observed in larval *occ* feeding on Douglas fir (Nealis 2012). It is possible that alleles that dictate diapause termination are fixed in *fum* and *bie*, allowing synchrony between larval emergence and primary host budburst. F1 hybrids between the taxa would receive a mixture of alleles from each parent, often leading to intermediate diapause termination, i.e., hybrid larvae emerge too late to feed on flushing white spruce but too early to feed on flushing subalpine fir or Engelmann spruce as these conifers flush at different times (compare Nienstaedt 1974 and Farnden 1994). However, larvae that feed on non-primary hosts (Furniss & Carolin 1977; Brown *et al.* 2008) confound this relationship, as these hosts likely allow alternative adult flight periods. This phenomenon may explain why adult F1 hybrids and other admixed individuals are collected during or near peak flight of either parental taxon (Lumley & Sperling 2011b; Figure 2-7), as larval F1 hybrids with intermediate developmental phenology would emerge from their hibernacula too late to be successful on *fum* host species, and much too early for success on *bie* host species.

Some localities experienced bimodal *bie* flight period during 2018 (Figure 2-8, localities 1, 2, 4, & 8). Early *bie* may represent quick-developing two-year individuals, i.e., their development is somewhat faster than typical *bie* collected in late July or August. Nealis (2005) determined that 1-5% of *bie* can have single-year development (one diapause period), and 10-50% can have three-year development (three diapause periods), depending on environmental

conditions. Our early fliers may represent individuals that did not achieve adulthood during the typical two-year period; instead, they may be "bet-hedgers" that underwent a third diapause. These *bie* would have had to spin third hibernacula just prior to achieving adulthood in their second year. This could result in earlier seasonal emergence in their third year, as their larval development would be nearly complete. Alternatively, early flying *bie* may have alleles that promote early emergence in their second year, although we did not see any evidence to support this.

Adult *C. occidentalis* from locality 1 experienced an unexpected bimodal flight period in 2018 (Figure 2-1). This may be explained by mean daily temperatures. A six-day period with mean daily temperatures of 20.5°C occurred just prior to the first peak in flight period (16 – 21 June, trap collected 24 June), which was directly followed by an 8-day period with mean daily temperatures of 13.0°C (25 June – 2 July). Liebhold & Volney (1984) observed a similar trend among *occ* and *C. retiniana* traps that were checked daily, allowing closer examination of the effect of temperature on individuals trapped. Futhermore, low temperatures decrease pheromone release rate (Sanders 1981) which may have affected trap catch.

Adult *bie* that were collected in early 2018 may have travelled from locations where *bie* exist in greater numbers. We collected *bie* individuals from localities 1, 2, and 10 during the same 10-day time periods in which we collected *fum* from localities 3–9 (Figure 2-8), suggesting western populations were a source of apparently quick-developing *bie.* Dobesberger, Lim, and Raske (1983) found that *fum* can travel >450 km if provided with proper conditions, and long-distance dispersals are routinely seen during outbreaks of *fum* in eastern North America (Greenbank, Schaefer, & Rainey 1980; Sturtevant *et al.* 2013). James *et al.* (2015) used microsatellite markers to determine that adult *fum* collected in Unitraps are not necessarily from

the same population as larvae feeding at the same location, suggesting that dispersal can significantly contribute to large-scale gene flow within the species. If *bie* dispersal behaviour is similar to that of *fum*, these findings suggest that *bie* traveled eastward from areas with higher host plant density in the Rocky Mountains and were intercepted in our Unitraps, which would explain their high frequency at localities 3 and 5 in 2018 (Figure 2-1, Figure 2-8).

Although temporal isolation was incomplete between taxa within 2018, there was near-complete isolation during 2017 in their overlapping range. We only identified two *bie* from the 2017 collection season in the overlap localities, both from locality 6 (Figure 2-8). Overall, we find that most of the contact between adult *fum* and *bie* may occur every second year, when the majority of *bie* have achieved adulthood. The combination of seasonal and between-year temporal isolation probably contributes to the continued maintenance of their genomic integrity, but our data suggest there are other ecological mechanisms that must also contribute to the genomic integrity of *fum* and *bie*. It is possible that other postmating isolation mechanisms, such as F2 hybrid breakdown, help to maintain the taxa.

Association of Temporal Isolation and Accumulated Degree-days

Calendar date better explained the temporal isolation between *fum* and *bie* than did degree-day accumulation ($r = 0.67$ *versus* $r = 0.41$; Figure 2-9). Our modelling assumes that the individuals we collected developed within the localities, although some may have dispersed (see previous section). Variance in flight period may have been better explained by degree-day accumulation if our collections had occurred more frequently; we could only correlate number of individuals collected with degree-days accumulated for each 10-day period. Also, our degree-day analysis assumes linear development, i.e. equal development rate at any temperature above 5.5°C

(Wilson & Barnett 1983). Spruce budworm development rate slightly varies with temperature (Bean 1961, Régnière *et al.* 2012), so more complex models may be required to elucidate the relationship between environmental effects and temporal isolation between these taxa. Additionally, development rate and the date at which larvae begin accumulating degree-days probably vary between or within populations. There is some evidence that the degree-days required to achieve adulthood vary across the range of *fum* (Volney & Fleming 2000, Candau *et al.* 2019), which may affect overlap in flight periods when dispersing spruce budworms come into contact.

Isolation Between and Within Subspecies of C. occidentalis

Although our study was not designed to evaluate the genomic integrity of *bie* and *occ*, we found two genetically diverged clusters at localities 1 and 2 (Figure 2-8). Recent studies using genome-wide SNPs found that *bie* and *occ* have a clinal relationship (Blackburn *et al.* 2017; Brunet *et al.* 2017), so we did not expect to find evidence of distinct genetic clusters of *C. occidentalis* within any single locality. It is possible that some of the collected individuals were long-range dispersers. However, individuals from localities 1 and 2 have a greater signal of intermediacy between *bie* and *occ* than do those from locality 10, which does suggest a clinal relationship between these taxa along the Rocky Mountain range (Figure 2-4). Clearly, more work is required to understand the relationship between *bie* and *occ*.

There was no evidence of biennial cohorts within *bie*, as adults collected in 2017 and 2018 were assigned to the same genetic cluster (Figure 2-5). Although it is possible that differentiation between these two cohorts exists, it is likely very weak. Adult *bie* have wide-scale emergence every second year upon achieving adulthood, but substantial flight is seen both in

even and odd years in different locations across their range (Zhang & Alfaro 2002, 2003). Given that *fum* can travel >450 km (Dobesberger *et al.* 1983) and a small proportion of *bie* have one- or three-year development (Nealis 2005), there is likely enough exchange between cohorts to erase any genetic differentiation between even- and odd-year cohorts. Further, both allochronic cohorts would face near-identical if not identical selection pressure (Gradish, Keyghobadi, Sperling, & Otis 2019), so most differentiation would be derived from genetic drift and probably slow to accumulate. Finally, our data filtering may have removed any signal of differentiation, such as by the removal of rare alleles through stringent minor allele frequency thresholds.

Adaptive SNPs and Preliminary Genomic Architecture

Some of the high-loading SNPs that contribute to the *bie* and *occ* genetic clusters are probably associated with diapause regulation (Figure 2-10; Table 2-7; Table 2-8), as there are few consistent life-history differences between these taxa other than diapause (Harvey 1967; Nealis 2005). Two of the six highest loading SNPs were associated with genomic regions that encode heat shock proteins (Table 2-7), which are synthesized by insects during the diapause period (Denlinger 2002; King & McRae 2015). These proteins allow the insect to tolerate stressful environmental changes during diapause (King & McRae 2015), possibly acting as a cryoprotectant (Denlinger 2002). Blackburn *et al.* (2017) also found one high-loading SNP associated with a heat shock gene that contributes to *bie* and *occ* clustering. Perhaps a handful, or even one of these heat shock associated SNPs are what causes fixation of the second diapause trait in *bie* (Nealis 2005). Other SNPs were associated with a gene for cyclic nucleotide phosphodiesterase (Table 2-7, SNP 631), which is used for hydrolysis of cAMP-specific cyclic phosphates to non-cyclic phosphates and thereby regulates many biological processes (Bender &

Beavo 2006), or a reverse transcriptase gene that could represent an inactivated retrotransposon, as such elements have been found in *fum* (Table 2-1, SNP 1342; Wang, Young, & Hickey 1995). Further investigation of these SNPs would aid our understanding of genetic differences between these taxa.

We found little evidence of linkage blocks contributing to isolation of *bie* and *occ*. By filtering for linkage disequilibrium using the *thin* function of VCFtools, we may have erased the any signal of linkage between SNPs. Further, we did not find many matches between our reference genome scaffolds and the current linkage map (Table 2-8; Picq *et al.* 2018). Of the three matches we did find (among 24 SNPs), two were located on linkage group 8. This may be evidence of a chromosomal island of differentiation, but much more work must be done to determine the genetic architecture of these taxa.

Evolution of C. o. biennis

The evolution of *bie* is likely to have occurred through adaptation of a cohort of *occ*-like ancestors to higher altitudes through fixation of alleles at diapause-associated genes, resulting in an obligatory second diapause. Second diapause is found in low proportions in *occ* (Harvey 1967; Nealis 2005), so adaptive selection on this trait could allow its proliferation. Second diapause can also be induced through manipulation of environmental conditions in *fum* (McMorran 1972), and naturally occurring two-year *fum* have been documented across their range (Harvey 1961; Candau *et al.* 2019), demonstrating that fixation of the character is possible. Second diapause causes slower development rates, allowing exploitation of brief host susceptibility windows and/or avoidance of low-quality environmental conditions. If *bie* evolved in this manner, it could be considered an allochronic speciation event.

In contrast to allochronic speciation, it may be that the initial genetic differentiation between *bie* and *occ* was driven by host specialization in addition to temporal shifts. Blackburn *et al.* (2017) found that some adaptive SNPs between these taxa were related to performance on conifer host(s). Fixation of the second diapause character could occur with or without a host shift, as conifer-feeding *Choristoneura* species are already polyphagous on various spruce and fir hosts (Furniss & Carolin 1977; Brown *et al.* 2008). More work is clearly required to elucidate the causal mechanisms of adaptive evolution in *C. occidentalis*.

Conclusion

We found that temporal isolation may aid in reducing hybridization between *Choristoneura fumiferana* and *Choristoneura occidentalis biennis*, in part driven by degree-day accumulation. We also found that there is nonetheless enough overlap in flight period between these taxa to allow hybridization. However, F1 hybrids were seen in lower proportions than expected, considering the extent of temporal overlap at sympatric localities. We also documented a range extension >100 km toward the east for *C. o. biennis*, from near Edson, Alberta to Elk Island National Park. Finally, we found no evidence for differentiation between biennial cohorts of *C. o. biennis*. We therefore suggest that other mechanisms play major roles in reducing gene flow between these taxa. We recommend further exploration of the ecological relationships between *C. fumiferana* and *C. o. biennis* to better understand how they maintain their genomic integrity.

Table 2-1. Calendar dates for all 2017 field collections. Locality numbers are as in Figure 1. Unitraps were installed during visit 1 (V1) and removed on the final date indicated (V7 or V8).

Locality	Larval Sampling	V1	V2	V3	V4	V5	V6	V7	V8
1. Clearwater, BC	157, 168	168	178	188	198	208	218	228	237
2. Vavenby, BC	158, 167	167	177	187	197	207	217	227	236
3. Wildhorse Lake, AB	148*, 158, 168	168	178	188	198	208	218	228	-
4. Obed Lake, AB	148*, 158, 168	168	178	188	198	208	218	228	-
5. Pembina Forks, AB	146*, 156, 166	166	176	186	196	206	216	226	229
6. Nordegg, AB	146*, 156, 166	166	176	186	196	206	216	226	229
7. Crimson Lake, AB	145*, 155, 165	165	175	185	195	205	215	225	229
8. Red Lodge, AB	145*, 155, 165	165	175	185	195	205	215	225	229
9. nr. Elk Island, AB	144*, 154, 164	164	174	184	194	204	214	224	235

* Current-year buds not visible or very small and therefore not counted

Table 2-2. Calendar dates for all 2018 field collections. Locality numbers are as in Figure 1. No larval sampling was performed. Unitraps and HOBO data loggers were installed during visit 1 (V1) and removed on the final visit (V10), except at Elk Island (*) which had a delayed start for the HOBO logger.

Locality	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
1. Clearwater, BC	155	165	175	186	195	205	215	225	235	245
2. Vavenby, BC	155	165	175	186	195	205	215	225	235	245
3. Wildhorse Lake, AB	156	166	176	187	196	206	216	226	236	246
4. Obed Lake, AB	156	166	176	187	196	206	216	226	236	246
5. Pembina Forks, AB	154	164	174	184	194	204	214	224	234	244
6. Nordegg, AB	154	164	174	184	194	204	214	224	234	244
7. Crimson Lake, AB	153	163	173	183	193	203	213	223	233	243
8. Red Lodge, AB	153	163	173	183	193	203	213	223	233	243
9. nr. Elk Island, AB	152	162	172 *	182	192	202	212	222	232	242
10. McBride, BC	156	166	176	187	196	206	216	226	236	246

Table 2-3. Summary of weather stations near each locality. Distance between station and locality calculated using a line between the two points. Distances are rounded to nearest km. Closest weather station match in bold, determined using Wilcoxon-Mann-Whitney test.

Locality	Nearest Weather Station(s)	Distance (km)	Database, URL
1. Clearwater, BC	Clearwater Auto	7 km	Government of Canada Historical Climate Data, http://climate.weather.gc.ca/historical_data/search_historic_data_e.html
2. Vavenby, BC	Vavenby	14 km	Government of Canada Historical Climate Data
3. Wildhorse Lake, AB	Entrance Auto	13 km	Alberta Climate Information Service, https://agriculture.alberta.ca/acis/alberta-weather-data-viewer.jsp
4. Obed Lake, AB	Edson	46 km	Alberta Climate Information Service
	Entrance Auto	41 km	Alberta Climate Information Service
	Schwartz Creek Auto	45 km	Alberta Climate Information Service
5. Pembina Forks, AB	Elk River Auto	51 km	Alberta Climate Information Service
	Luscar Creek	49 km	Alberta Climate Information Service
	Schwartz Creek Auto	50 km	Alberta Climate Information Service
6. Nordegg, AB	Nordegg	2 km	Alberta Climate Information Service
7. Crimson Lake, AB	Rocky Mountain House	8 km	Alberta Climate Information Service
8. Red Lodge, AB	Dickson Dam	12 km	Alberta Climate Information Service
9. nr. Elk Island, AB	Elk Island Nat Park	7 km	Alberta Climate Information Service
10. McBride, BC	McBride Mountain Station	8 km	Pacific Climate Impacts Consortium - BC Station Data, https://data.pacificclimate.org/portal/pcds/map/

Table 2-4. Summary of HOBO data logger and weather station temperature records used to calculate temperature correction factors. Median temperature refers to the period that loggers were present at the locality. WMW = Wilcoxon-Mann-Whitney test. Bold p-value indicates significant difference between logger and weather station temperatures. r = effect size.

Locality	Distance between locality & weather station	Daily High Temperatures			Daily Low Temperatures		
		Median Temperature (°C)		WMW test results	Median Temperature (°C)		WMW test results
		locality	station		locality	station	
1	7 km	22.9	26.5	U = 2856, **p = 0.0013**, r = 0.23	11.3	10.0	U = 5162, **p < 0.001**, r = 0.24
2	14 km	21.1	25.5	U = 2229, **p < 0.001**, r = 0.34	9.1	10.0	U = 3233, p = 0.097, r = 0.098
3	13 km	19.7	22.1	U = 2890, **p = 0.0037**, r = 0.20	8.5	7.1	U = 4914, **p = 0.0021**, r = 0.22
4	45 km	22.5	21.1	U = 4264, p = 0.25, r = 0.052	7.7	8.4	U = 3392, p = 0.16, r = 0.076
5	49 km	21.7	18.6	U = 5043, **p = 0.0016**, r = 0.22	3.9	3.6	U = 4096, p = 0.69, r = 0.038
6	2 km	21.6	20.8	U = 4111, p = 0.48, r = 0.0037	5.1	5.4	U = 3698, p = 0.61, r = 0.02
7	8 km	23.1	22.9	U = 3950, p = 0.98, r = 0.15	7.1	6.0	U = 4685, **p = 0.035**, r = 0.13
8	12 km	24.5	24.5	U = 3863, p = 0.78, r = 0.057	7.5	9.4	U = 2488, **p < 0.001**, r = 0.31
9	7 km	24.2	24.4	U = 2519, p = 0.56, r = 0.012	10.2	10.3	U = 2389, p = 0.97, r = 0.16
10	8 km	18.2	12.9	U = 6077, **p < 0.001**, r = 0.48	9.9	5.9	U = 5969, **p < 0.001**, r = 0.46

Table 2-5. Summary of larval sampling, with mean number of buds per branch and number of branches, by locality and tree species. [1] = one larva collected. [2] = two larvae collected.

Locality	White spruce		Douglas fir	
	Mean Buds / Branch (± SD)	Total Branches	Mean Buds / Branch (± SD)	Total Branches
1. Clearwater, BC	30.1 ± 6.7 [1]	20	25.6 ± 11.8	20
2. Vavenby, BC	27.2 ± 8.1	10	26.6 ± 8.9	30
3. Wildhorse Lake, AB	31.4 ± 12.1	40	-	-
4. Obed Lake, AB	29.3 ± 10.2	40	-	-
5. Pembina Forks, AB	31.8 ± 16.7	40	-	-
6. Nordegg, AB	43.1. ± 17.0	40	-	-
7. Crimson Lake, AB	39.8 ± 16.2	40	-	-
8. Red Lodge, AB	40.3 ± 15.6 [2]	40	-	-
9. nr. Elk Island, AB	41.0 ± 15.7 [1]	40	-	-
all localities	**35.9 ± 15.2**	**310**	**26.2 ± 8.9**	**50**

Table 2-6. Summary of estimates of hybridization rates between *fum* and subspecies of *C. occidentalis.*

Location	Hybridization Rate Between *fum* and *bie* + *occ*	
	Microsatellite and mtDNA Mismatch	*structure* F1s *
Cypress Hills, Alberta [1]	**7.3%** (21 of 286)	-
Crowsnest Pass, Alberta [2]	**7.0%** (3 of 41)	-
Canadian Rocky Mountains and Western Alberta [3]	-	**0.6%** (3 of 529)
range overlap between *fum* and *bie* + *occ* [3]	-	**1.1%** (3 of 281)
range overlap between *fum* and *bie* + *occ* [4]	-	**2.9%** (5 of 175)

[1] Lumley & Sperling 2011b, [2] Brunet *et al.* 2013, [3] Brunet *et al.* 2017, [4] Present study
* approximately 50% membership to both *fum* and *bie* + *occ* genetic clusters in *structure*

Table 2-7. Summary of BLASTX search results for SNPs with greatest contributions to PCA clustering of bie and occ (Figure 2-4, PC1).

SNP	Top BLASTX Protein Match	*E* value	Putative Function	Linkage Group Location *
199	uncharacterized protein LOC111361554	9.00E-77	-	Linkage Group 8
556	hypothetical protein B5X24_HaOG209322	1.00E-48	-	No match
631	cAMP-specific 3',5'-cyclic phosphodiesterase isoform X7	3.00E-04	Cyclic Nucleotide Phosphodiesterase Activity	No match
656	heat shock 70 kDa protein 4	1.00E-51	Cryoprotectant During Diapause	No match
1342	reverse transcriptase	2.00E-59	Virus Replication	Linkage Group 6
1347	97 kDA heat shock protein isoform X1	2.00E-50	Cryoprotectant During Diapause	No match

* *Choristoneura fumiferana* linkage map from Picq *et al.* 2018

Table 2-8. Full summary of BLASTX search results for SNPs with greatest contribution to PC1 of PCA between *bie* and *occ* (Figure 2-10).

SNP	Top BLASTX Protein Match	*E* value	Location on Linkage Map *
91	hypothetical protein B5X24_HaOG204443	2.00E-32	No match
122	hypothetical protein evm_005519	7.00E-13	No match
153	nuclear receptor coactivator 2-like	5.00E-39	No match
157	bunched class 2 isoform	0.00E+00	No match
176	hypothetical protein AB894_15325	2.00E-55	No match
198	integrator complex subunit 10	0.00E+00	Linkage Group 8
199	uncharacterized protein LOC111361554	9.00E-77	Linkage Group 8
215	uncharacterized protein LOC113494593	5.00E-101	No match
224	unknown	2.30E+00	No match
421	myc protein	2.00E-62	No match
442	Low Quality Protein: unconventional myosin-la-like	2.00E-30	No match
556	hypothetical protein B5X24_HaOG209322	1.00E-48	No match
631	cAMP-specific 3',5'-cyclic phophodiesterase isoform X7	3.00E-04	No match
656	heat shock 70 kDa protein 4	1.00E-51	No match
657	uncharacterized protein LOC113511970	5.00E-55	No match
668	uncharacterized protein LOC113236272	5.00E-67	No match
881	hypothetical protein B5X24_HaOG204513	1.00E-46	No match
954	No match	-	No match
1049	uncharacterized protein LOC113508709	7.00E-55	No match
1102	No match	-	No match
1211	uncharacterized protein LOC111352608	7.00E-14	No match
1226	putative uncharacterized protein DDB_G0282133	8.00E-05	No match
1342	reverse transcriptase	2.00E-59	Linkage Group 6
1347	97 kDA heat shock protein isoform X1	2.00E-50	No match

* *Choristoneura fumiferana* linkage map from Picq *et al.* 2018

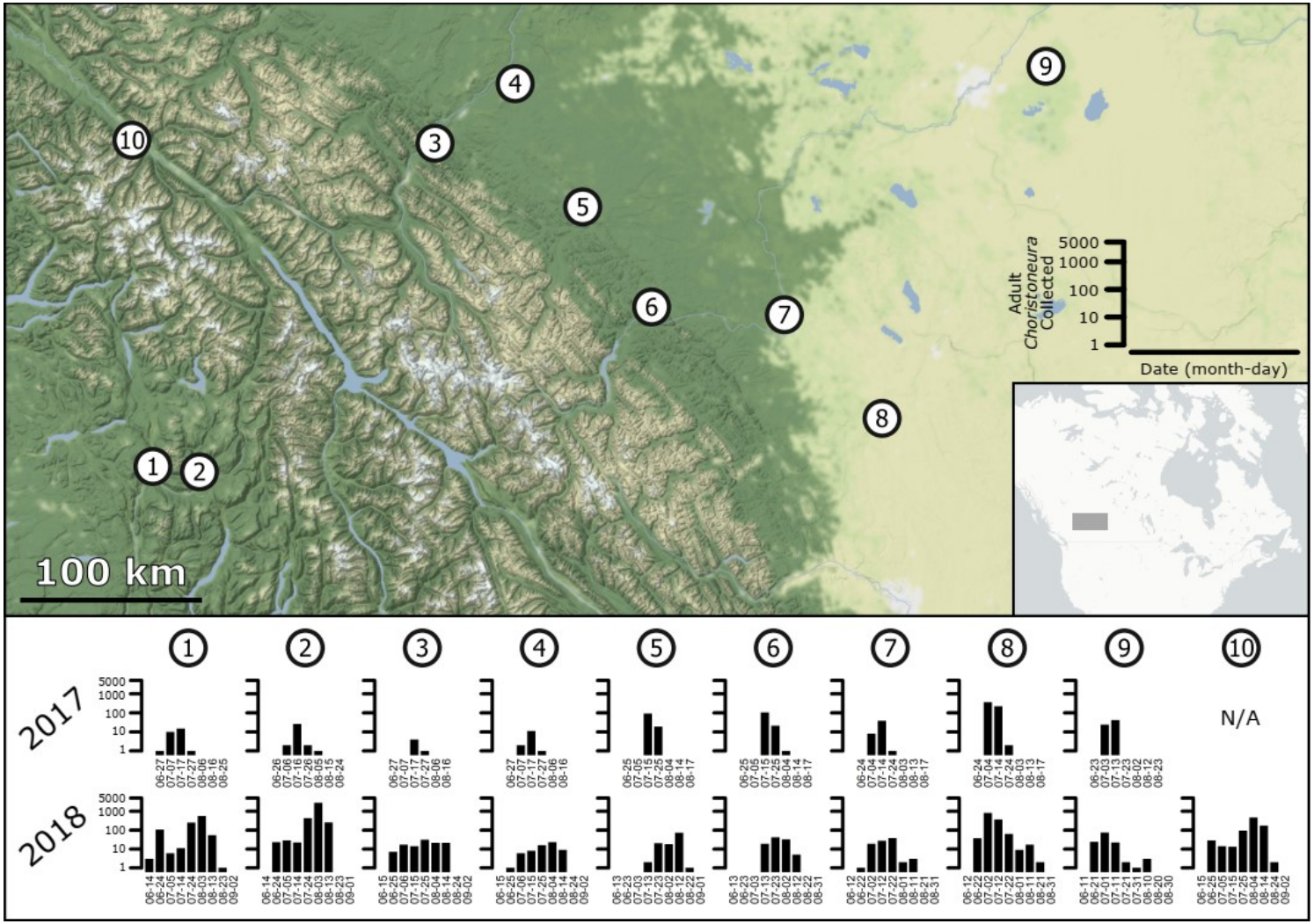

Figure 2-1. Map of study area showing localities and phenology of collected adult spruce budworm species. Locality names are provided in Table 2-2. Map prepared in QGIS version 3.6.0 and base maps obtained from https://github.com/stamen/terrain-classic and https://github.com/CartoDB/basemap-styles.

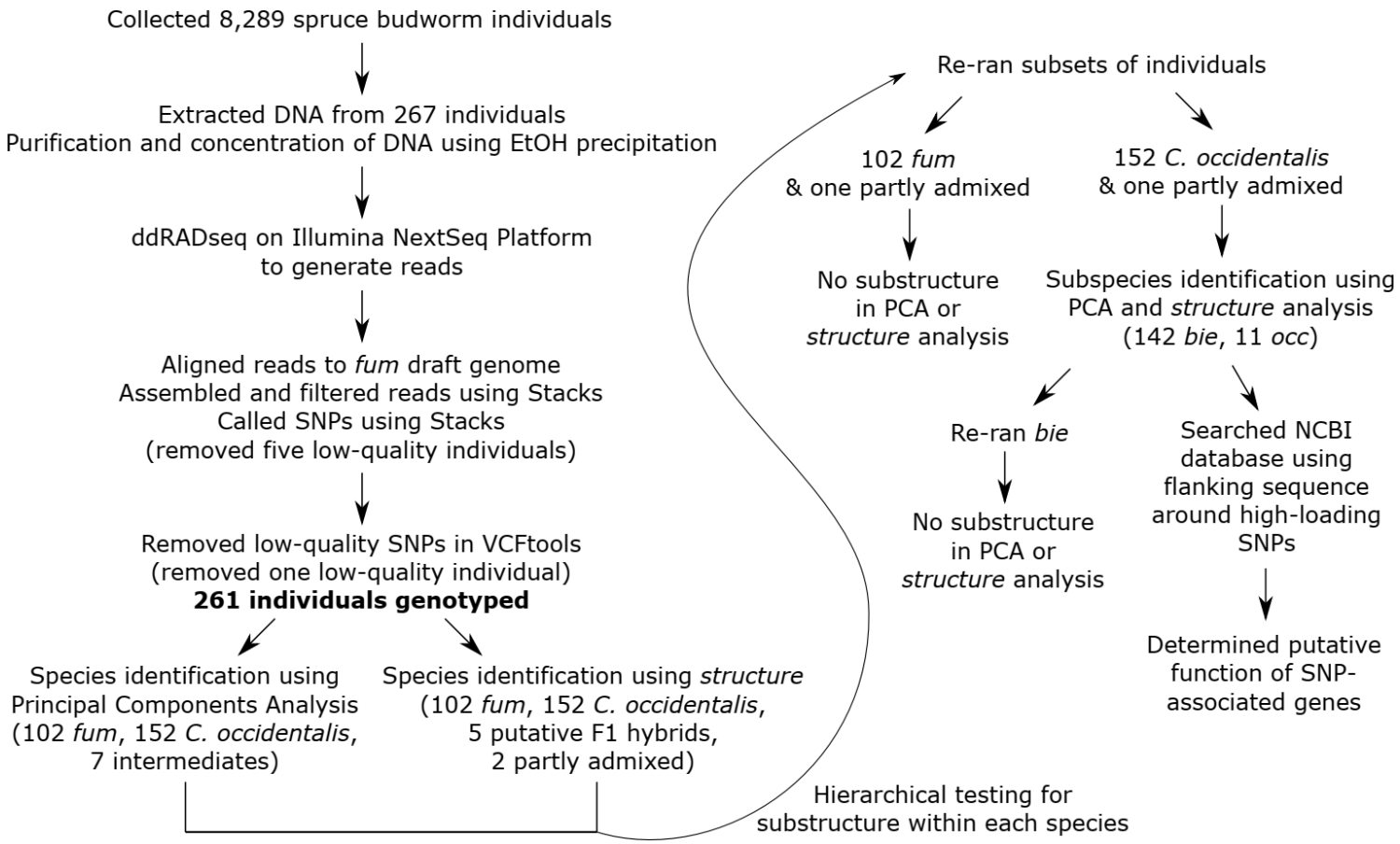

Figure 2-2. A flowchart of our laboratory and bioinformatic methods.

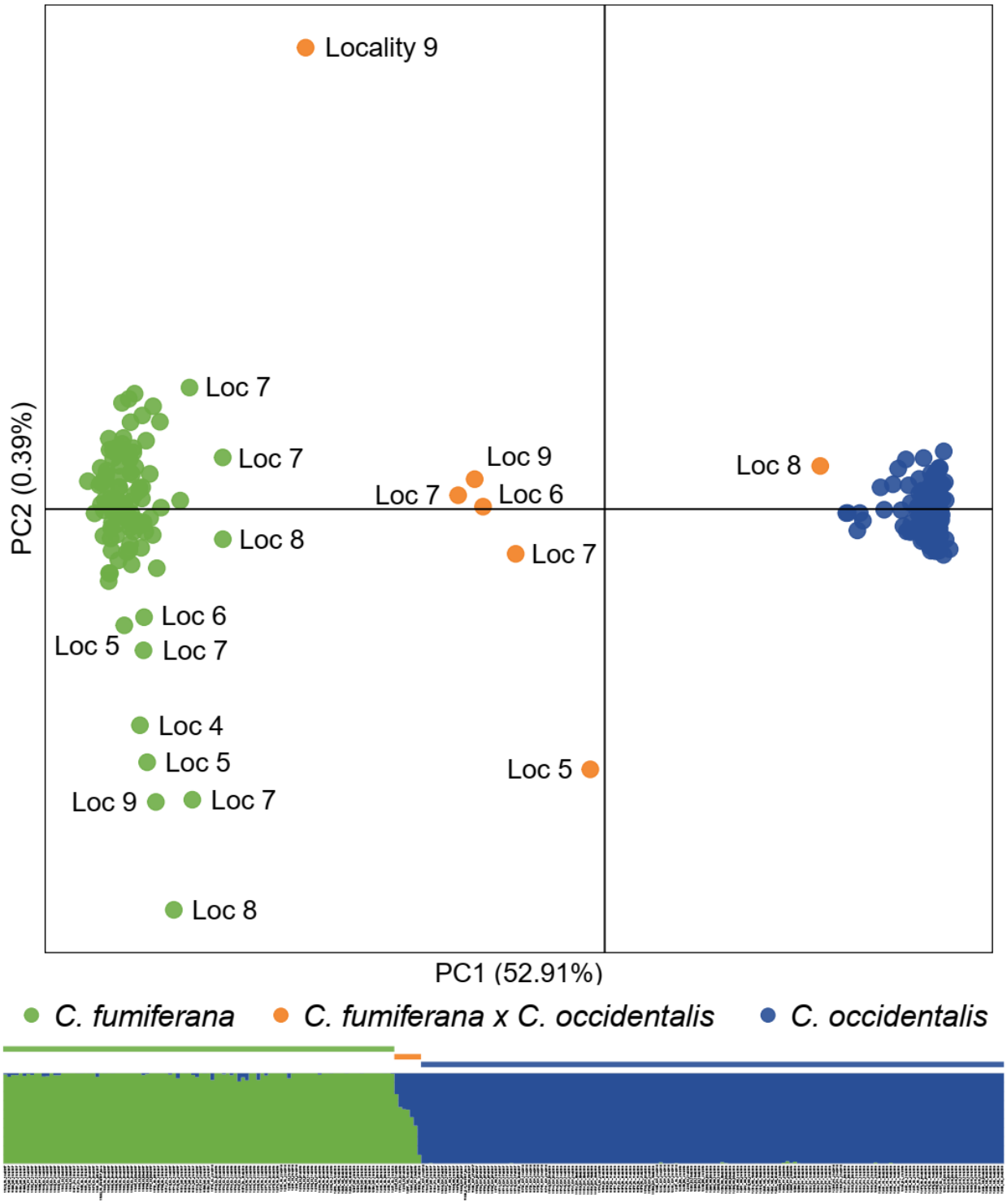

Figure 2-3. Principal component analysis and *structure* barplot of SNPs for all 261 spruce budworms. Individuals that had ≤90% assignment to either cluster of the *structure* analysis are in the centre of the barplot and labelled "*C. fumiferana x C. occidentalis*".

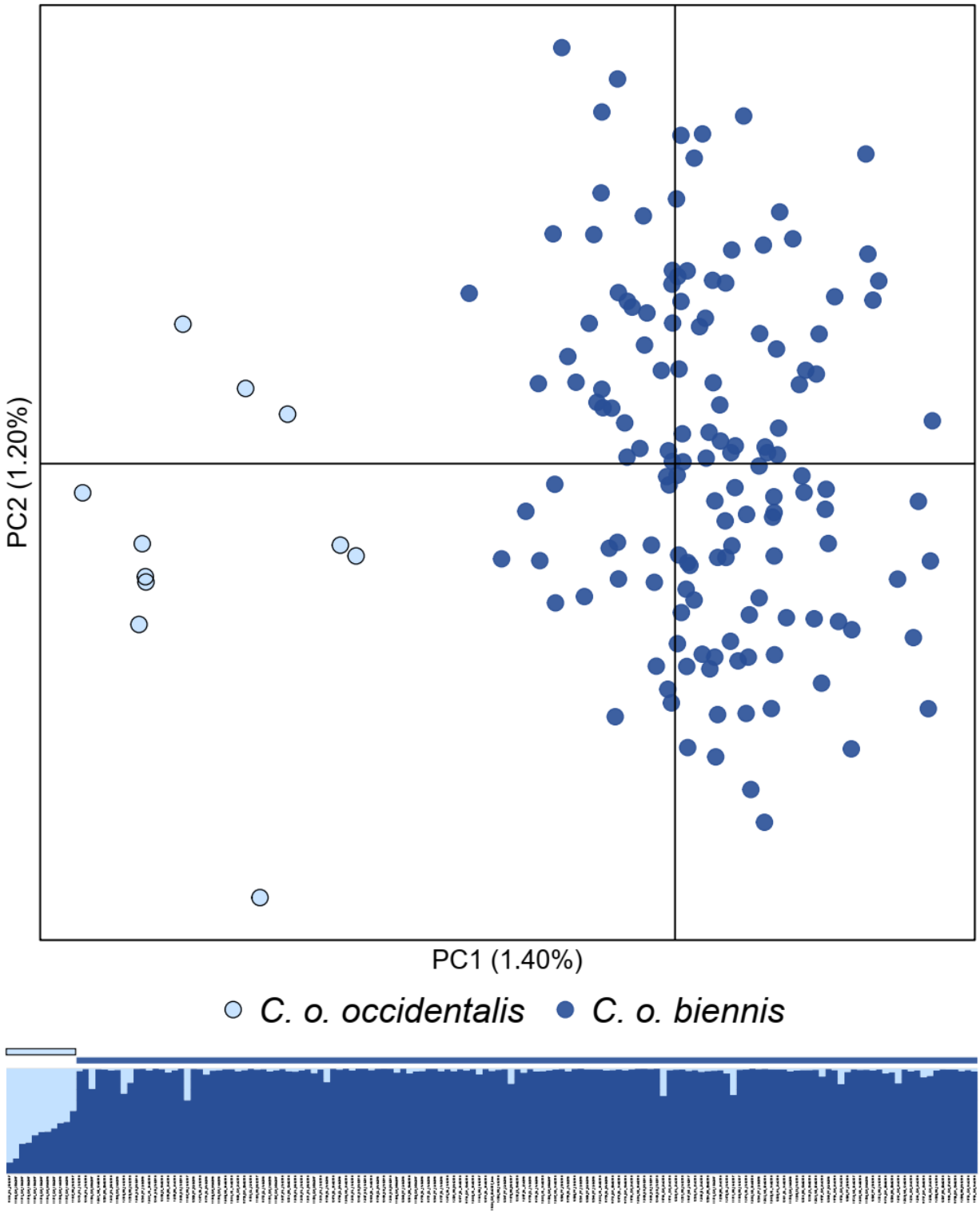

Figure 2-4. Principal component analysis and *structure* barplot of SNPs in 153 *C. occidentalis* specimens. Individuals that had <60% assignment to the major cluster (*bie*) in the *structure* plot are in the centre of the barplot and labelled "*C. o. occidentalis*".

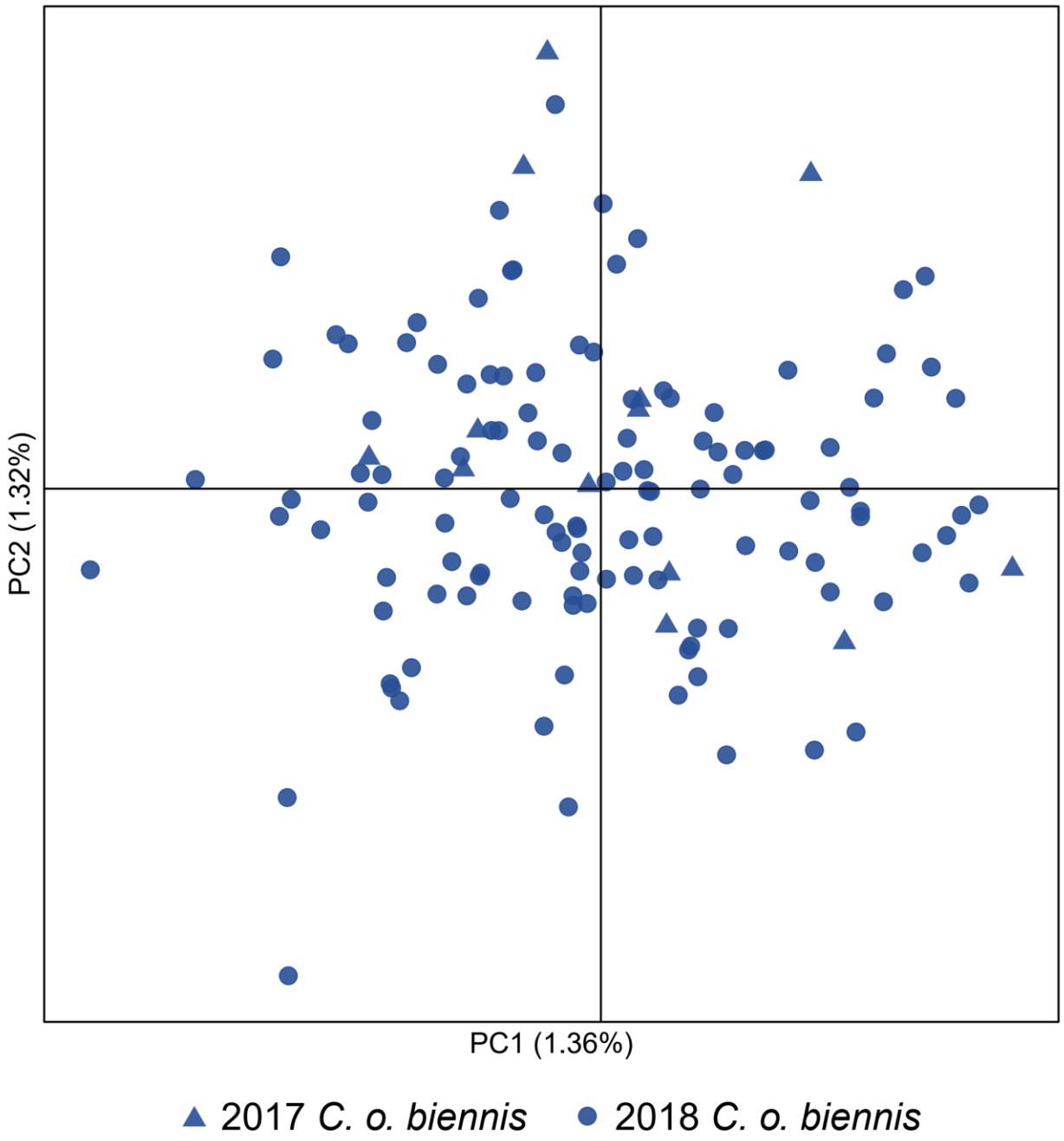

Figure 2-5. Principal component analysis of SNPs in 132 *bie* specimens.

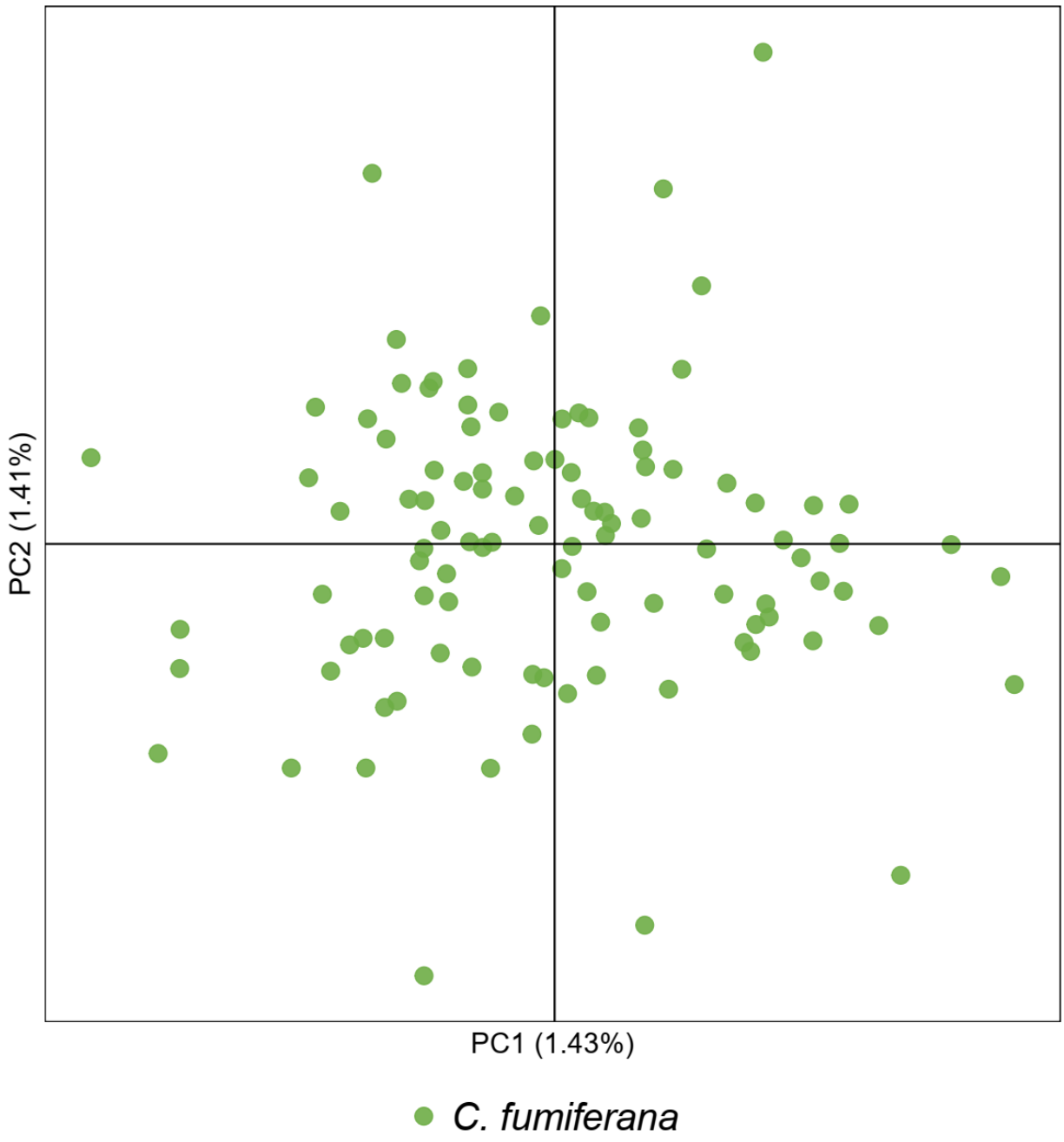

Figure 2-6. Principal component analysis of SNPs in 103 *fum* specimens.

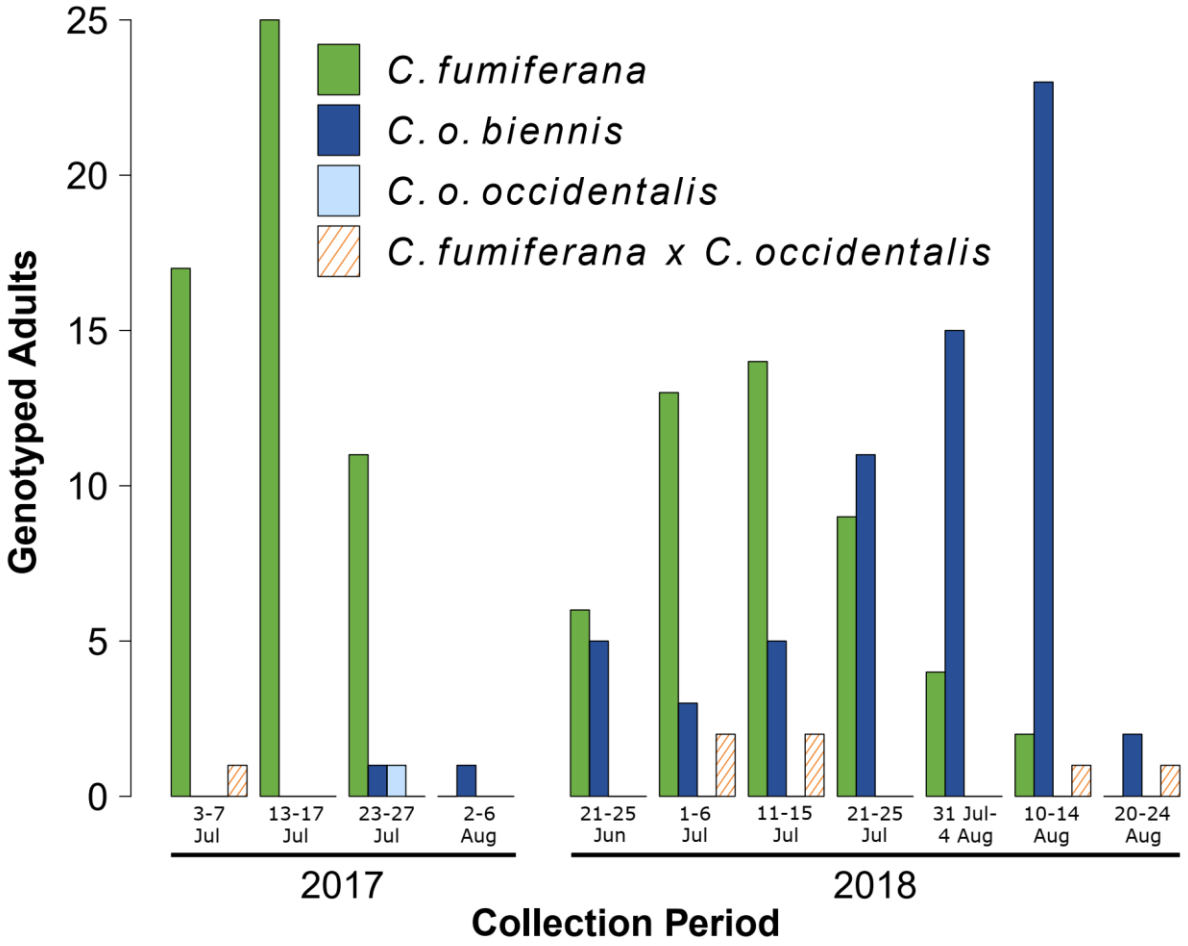

Figure 2-7. Histogram of genotyped spruce budworm moth species from "overlap localities" 3–9 (Figure 2-1). Dates reflect day of trap catch collection, i.e. moths were trapped between this period and the previous trap collection 10 days earlier.

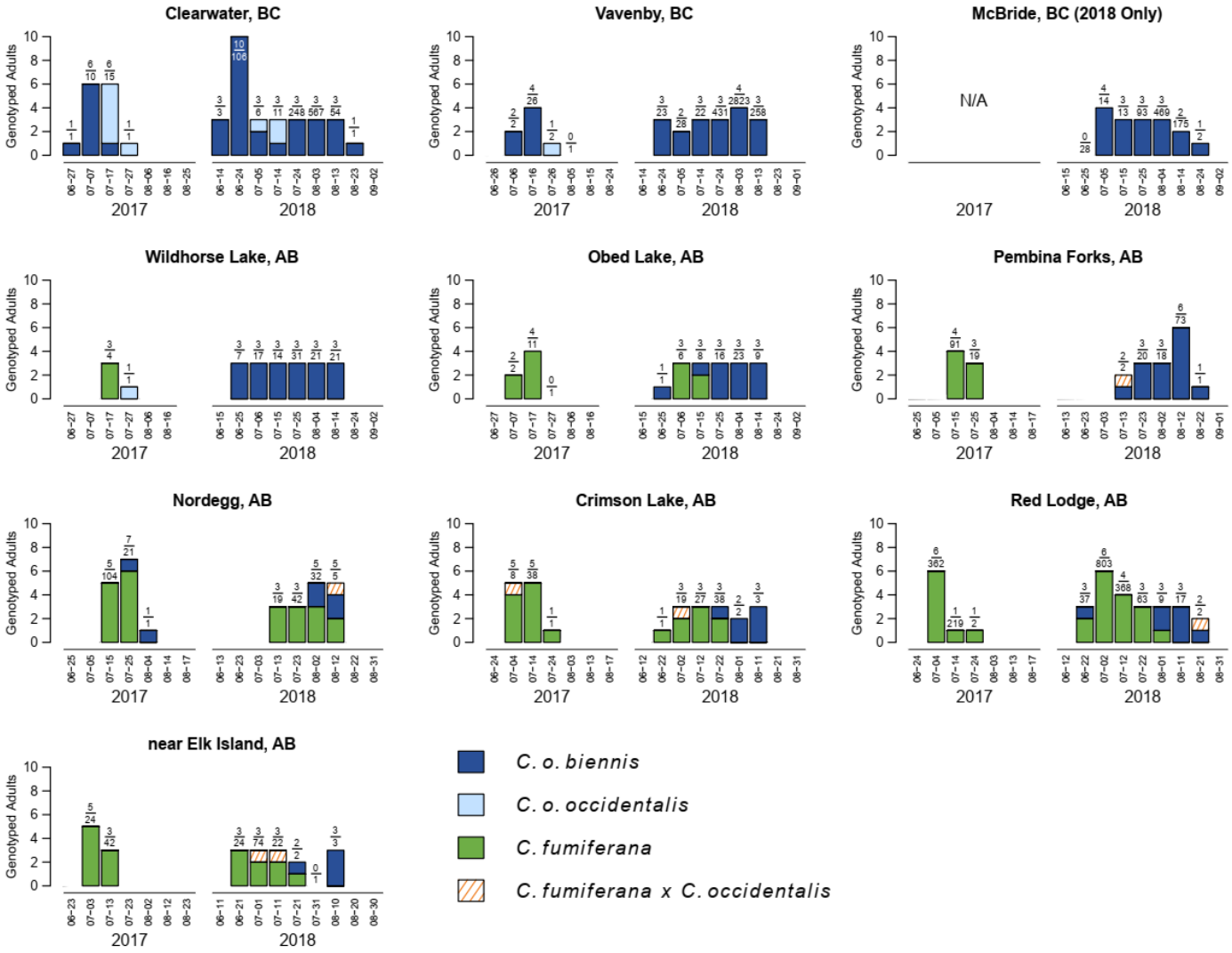

Figure 2-8. Histogram series of genotyped adults collected from each locality. X-axis dates listed as month-day. Dates reflect day of trap catch collection. Proportions above bars denote subsampling effort.

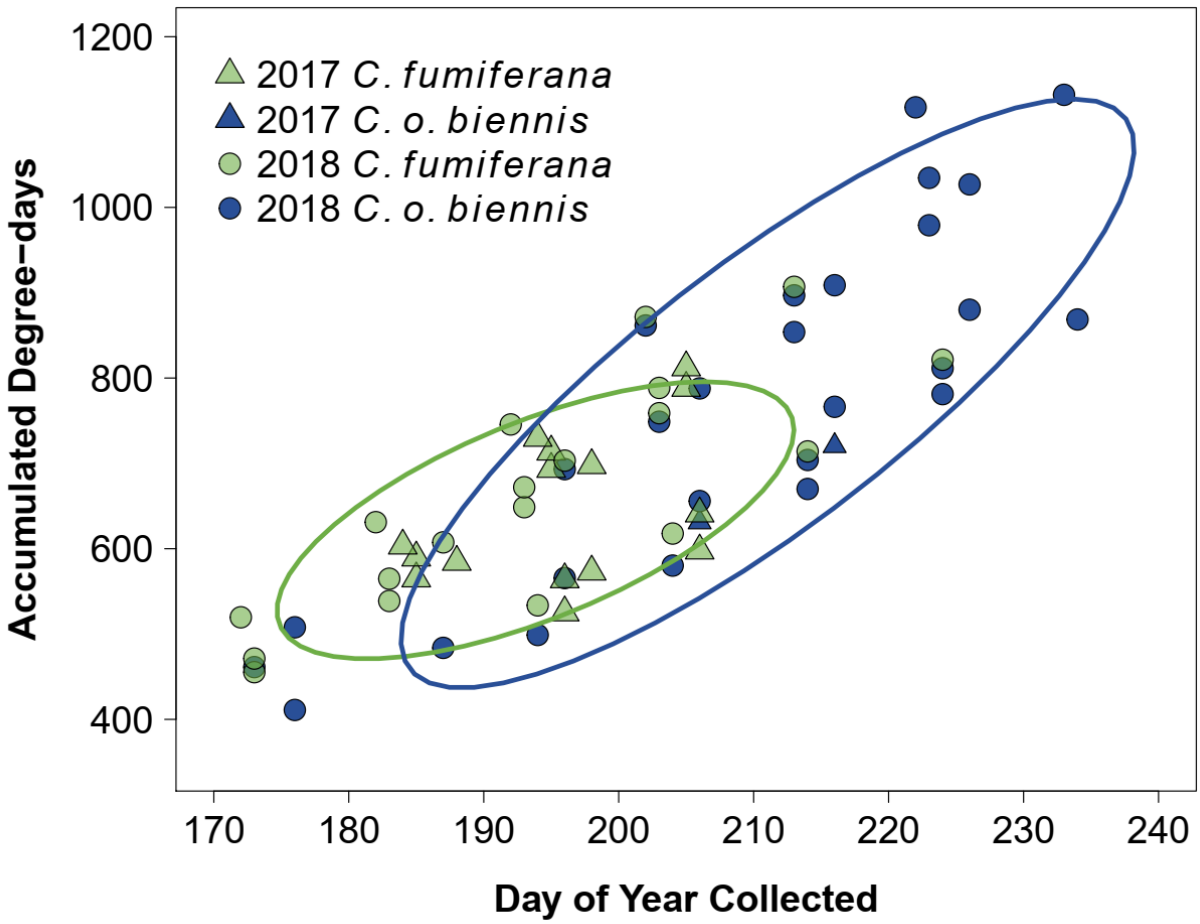

Figure 2-9. Association between calendar day that *fum* and *bie* were collected across the "overlap localities" 3-9, with local degree-day accumulation calculated using the single triangulation method (Lindsey & Newman 1956). Each point denotes ≥1 individual that was collected and genotyped. 80% ellipses encompass all genotyped individuals of these taxa from these localities and are centred around the median calendar day collected and median number of corrected, accumulated degree-days. Data for *bie* are shifted downward by 10 degree-days for easier visibility, but the *bie* ellipse has not been shifted.

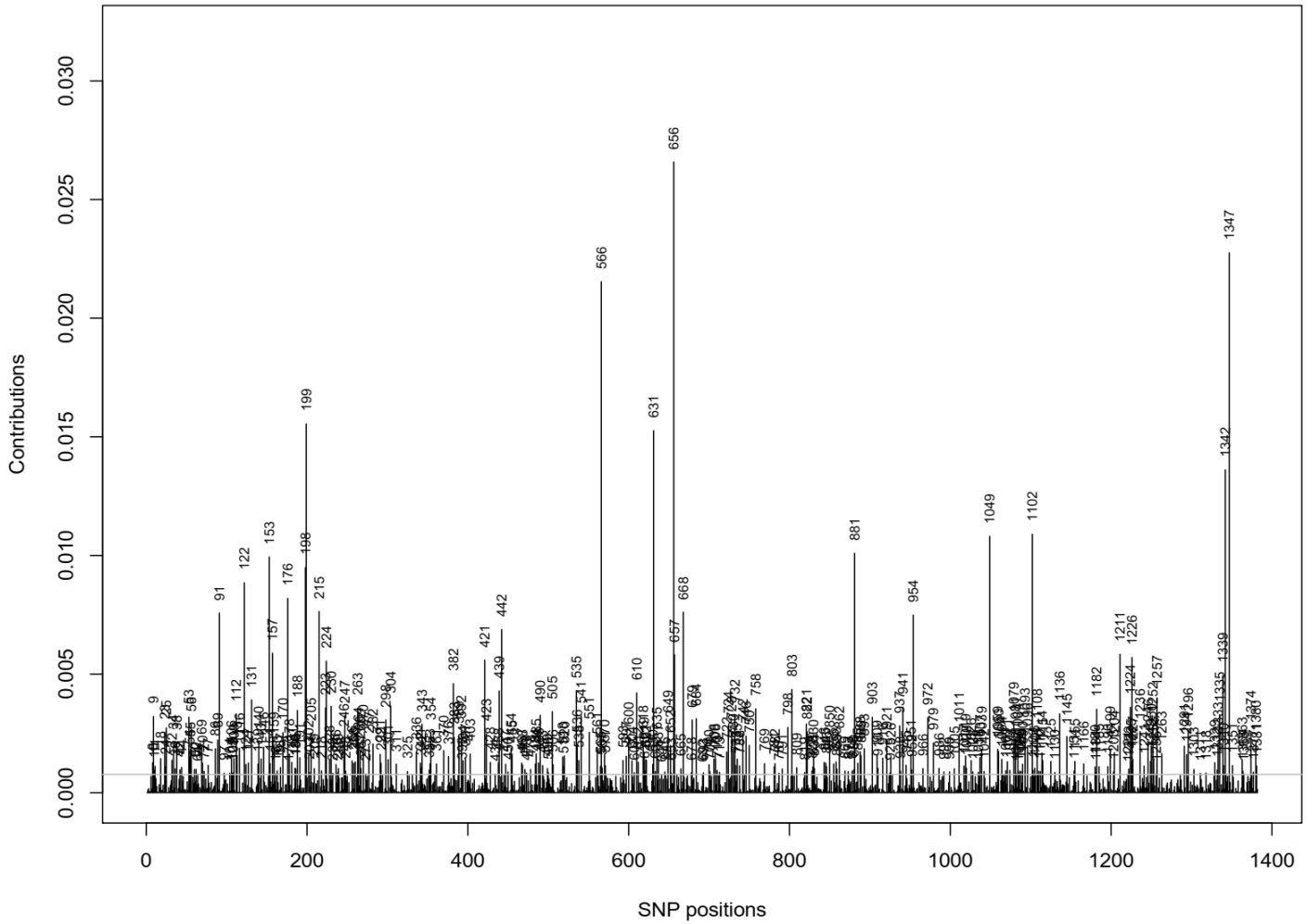

Figure 2-10. Loading plot of SNPs contributing to difference between *bie* and *occ* in PC1 of Figure 2-4. Plot prepared using *loadingplot* function of adegenet R package (Jombart & Ahmed 2011). BLASTX search results for SNPs with contribution ≥0.005 in Table 2-8.

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Appendix 2-1. Summary of all collected *Choristoneura*.

Locality (Fig 1 Label)	Location	Province	2017 Collected *Choristoneura*	2018 Collected *Choristoneura*	Total Collected *Choristoneura*
1	Clearwater	BC	28	996	1024
2	Vavenby	BC	31	3585	3616
3	Wildhorse Lake PRA	AB	5	111	116
4	Obed Lake PP	AB	14	63	77
5	Pembina Forks	AB	110	114	224
6	Nordegg	AB	126	98	224
7	Crimson Lake PP	AB	47	90	137
8	Red Lodge PP	AB	585	1299	1884
9	Near Elk Island NP	AB	67	126	193
10	McBride	BC	-	794	794
					8289

Appendix 2-2. Summary of genotyped *Choristoneura.* Removed = Y indicates the individual was removed from the dataset due to >30% missing genetic data, or because it was intentionally re-sequenced to check for run effects (labelled "reseq"). These individuals were not assigned University of Alberta Strickland Museum (UASM) numbers.

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
11625	-	Red Lodge PP	AB	TD Nelson	2017-07-14	51.9460	-114.2381	Adult	Unitrap	Male	Y
11632	391501	Nordegg	AB	TD Nelson	2017-07-15	52.4878	-116.0703	Adult	Unitrap	Male	N
11637	391502	Pembina Forks	AB	TD Nelson	2017-07-15	52.9693	-116.6182	Adult	Unitrap	Male	N
11643	3915034	Crimson Lake PP	AB	TD Nelson	2017-07-14	52.4506	-115.0148	Adult	Unitrap	Male	N
11645	391504	Vavenby	BC	TD Nelson	2017-07-16	51.6845	-119.6627	Adult	Unitrap	Male	N
11647	391505	Nordegg	AB	TD Nelson	2017-07-25	52.4878	-116.0703	Adult	Unitrap	Male	N
11654	391506	Wildhorse Lake PRA	AB	TD Nelson	2017-07-27	53.2719	-117.7909	Adult	Unitrap	Male	N
11658	391507	Clearwater	BC	TD Nelson	2017-07-27	51.7100	-120.0334	Adult	Unitrap	Male	N
11661	391508	Clearwater	BC	TD Nelson	2017-07-17	51.7100	-120.0334	Adult	Unitrap	Male	N
11628	391509	Red Lodge PP	AB	TD Nelson	2017-07-14	51.9460	-114.2381	Adult	Unitrap	Male	N
11629	391510	Nordegg	AB	TD Nelson	2017-07-15	52.4878	-116.0703	Adult	Unitrap	Male	N
11630	391511	Nordegg	AB	TD Nelson	2017-07-15	52.4878	-116.0703	Adult	Unitrap	Male	N
11631	391512	Nordegg	AB	TD Nelson	2017-07-15	52.4878	-116.0703	Adult	Unitrap	Male	N
11633	391513	Nordegg	AB	TD Nelson	2017-07-15	52.4875	-116.0708	Adult	Unitrap	Male	N
11634	391514	Pembina Forks	AB	TD Nelson	2017-07-15	52.9689	-116.6176	Adult	Unitrap	Male	N
11635	391515	Pembina Forks	AB	TD Nelson	2017-07-15	52.9689	-116.6176	Adult	Unitrap	Male	N
11639	391516	Pembina Forks	AB	TD Nelson	2017-07-15	52.9689	-116.6184	Adult	Unitrap	Male	N
11640	391517	Crimson Lake PP	AB	TD Nelson	2017-07-14	52.4502	-115.0140	Adult	Unitrap	Male	N
11641	391518	Crimson Lake PP	AB	TD Nelson	2017-07-14	52.4504	-115.0141	Adult	Unitrap	Male	N
11653	391519	Pembina Forks	AB	TD Nelson	2017-07-25	52.9689	-116.6184	Adult	Unitrap	Male	N
11657	391520	Vavenby	BC	TD Nelson	2017-07-26	52.9689	-116.6184	Adult	Unitrap	Male	N
11660	391521	Crimson Lake PP	AB	TD Nelson	2017-07-24	52.4506	-115.0148	Adult	Unitrap	Male	N
11662	391522	Near Elk Island NP	AB	TD Nelson, S Bishop	2017-07-13	53.6355	-112.9354	Adult	Unitrap	Male	N
11665	391523	Near Elk Island NP	AB	TD Nelson, S Bishop	2017-07-13	53.6360	-112.9359	Adult	Unitrap	Male	N
11666	391524	Near Elk Island NP	AB	TD Nelson, S Bishop	2017-07-13	53.6355	-112.9369	Adult	Unitrap	Male	N
11667	391525	Vavenby	BC	TD Nelson	2017-07-16	51.6837	-119.6634	Adult	Unitrap	Male	N
11669	391526	Vavenby	BC	TD Nelson	2017-07-16	51.6845	-119.6627	Adult	Unitrap	Male	N
11671	391527	Wildhorse Lake PRA	AB	TD Nelson	2017-07-17	53.2719	-117.7909	Adult	Unitrap	Male	N
11672	391528	Wildhorse Lake PRA	AB	TD Nelson	2017-07-17	53.2719	-117.7901	Adult	Unitrap	Male	N
11673	391529	Obed Lake PP	AB	TD Nelson	2017-07-17	53.5535	-117.1541	Adult	Unitrap	Male	N
11674	391530	Obed Lake PP	AB	TD Nelson	2017-07-17	53.5539	-117.1548	Adult	Unitrap	Male	N
11677	391531	Red Lodge PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	51.9460	-114.2381	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
11678	391532	Crimson Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	52.4502	-115.0140	Adult	Unitrap	Male	N
11679	391533	Vavenby	BC	TD Nelson, BD Wingert, RLK French	2017-07-06	51.6845	-119.6627	Adult	Unitrap	Male	N
11680	391534	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7100	-120.0334	Adult	Unitrap	Male	N
11681	391535	Obed Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-07	53.5535	-117.1541	Adult	Unitrap	Male	N
11682	391536	Red Lodge PP	AB	TD Nelson	2017-06-04	-	-	Larva	Hand	Unknown	N
11685	-	Near Elk Island NP	AB	TD Nelson, WK Kellerman	2017-06-13	-	-	Larva	Hand	Unknown	Y
11686	391537	Red Lodge PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	51.9460	-114.2381	Adult	Unitrap	Male	N
11687	391538	Red Lodge PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	51.9458	-114.2385	Adult	Unitrap	Male	N
11688	391539	Red Lodge PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	51.9458	-114.2385	Adult	Unitrap	Male	N
11689	391540	Red Lodge PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	51.9457	-114.2390	Adult	Unitrap	Male	N
11690	391541	Red Lodge PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	51.9457	-114.2390	Adult	Unitrap	Male	N
11691	391542	Crimson Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	52.4502	-115.0140	Adult	Unitrap	Male	N
11692	391543	Crimson Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	52.4504	-115.0141	Adult	Unitrap	Male	N
11693	391544	Crimson Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	52.4504	-115.0141	Adult	Unitrap	Male	N
11694	391545	Crimson Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-04	52.4504	-115.0141	Adult	Unitrap	Male	N
11695	391546	Vavenby	BC	TD Nelson, BD Wingert, RLK French	2017-07-06	51.6845	-119.6627	Adult	Unitrap	Male	N
11696	391547	Clearwater	BC	TD Nelson	2017-06-27	51.7101	-120.0345	Adult	Unitrap	Male	N
11697	391548	Near Elk Island NP	AB	TD Nelson, BD Wingert	2017-07-03	53.6355	-112.9354	Adult	Unitrap	Male	N
11698	391549	Near Elk Island NP	AB	TD Nelson, BD Wingert	2017-07-03	53.6360	-112.9359	Adult	Unitrap	Male	N
11699	391550	Near Elk Island NP	AB	TD Nelson, BD Wingert	2017-07-03	53.6355	-112.9369	Adult	Unitrap	Male	N
11700	391551	Near Elk Island NP	AB	TD Nelson, BD Wingert	2017-07-03	53.6355	-112.9354	Adult	Unitrap	Male	N
11701	391552	Near Elk Island NP	AB	TD Nelson, BD Wingert	2017-07-03	53.6355	-112.9354	Adult	Unitrap	Male	N
11702	391553	Obed Lake PP	AB	TD Nelson, BD Wingert, RLK French	2017-07-07	53.5535	-117.1541	Adult	Unitrap	Male	N
11703	391554	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7100	-120.0334	Adult	Unitrap	Male	N
11704	391555	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7105	-120.0345	Adult	Unitrap	Male	N
11705	391556	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7105	-120.0345	Adult	Unitrap	Male	N
11706	391557	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7101	-120.0345	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
11707	391558	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7101	-120.0345	Adult	Unitrap	Male	N
11708	391559	Obed Lake PP	AB	TD Nelson	2017-07-17	53.5535	-117.1541	Adult	Unitrap	Male	N
11709	391560	Obed Lake PP	AB	TD Nelson	2017-07-17	53.5539	-117.1548	Adult	Unitrap	Male	N
11712	391561	Wildhorse Lake PRA	AB	TD Nelson	2017-07-17	53.2720	-117.7907	Adult	Unitrap	Male	N
11713	391562	Crimson Lake PP	AB	TD Nelson	2017-07-14	52.4506	-115.0148	Adult	Unitrap	Male	N
11714	391563	Crimson Lake PP	AB	TD Nelson	2017-07-14	52.4504	-115.0141	Adult	Unitrap	Male	N
11716	391564	Clearwater	BC	TD Nelson	2017-07-17	51.7105	-120.0345	Adult	Unitrap	Male	N
11717	391565	Clearwater	BC	TD Nelson	2017-07-17	51.7101	-120.0345	Adult	Unitrap	Male	N
11718	391566	Clearwater	BC	TD Nelson	2017-07-17	51.7101	-120.0345	Adult	Unitrap	Male	N
11719	391567	Clearwater	BC	TD Nelson	2017-07-17	51.7100	-120.0334	Adult	Unitrap	Male	N
11720	391568	Red Lodge PP	AB	TD Nelson	2017-07-24	51.9460	-114.2381	Adult	Unitrap	Male	N
11722	391569	Nordegg	AB	TD Nelson	2017-07-25	52.4878	-116.0703	Adult	Unitrap	Male	N
11723	391570	Nordegg	AB	TD Nelson	2017-07-25	52.4875	-116.0708	Adult	Unitrap	Male	N
11724	391571	Nordegg	AB	TD Nelson	2017-07-25	52.4875	-116.0708	Adult	Unitrap	Male	N
11725	391572	Nordegg	AB	TD Nelson	2017-07-25	52.4875	-116.0708	Adult	Unitrap	Male	N
11726	391573	Pembina Forks	AB	TD Nelson	2017-07-25	52.9689	-116.6184	Adult	Unitrap	Male	N
11727	391574	Pembina Forks	AB	TD Nelson	2017-07-25	52.9693	-116.6182	Adult	Unitrap	Male	N
11728	391575	Nordegg	AB	TD Nelson	2017-08-04	52.4878	-116.0703	Adult	Unitrap	Male	N
11730	391576	Nordegg	AB	TD Nelson	2017-07-25	52.4878	-116.0703	Adult	Unitrap	Male	N
11732	391577	Nordegg	AB	TD Nelson	2017-07-25	52.4875	-116.0708	Adult	Unitrap	Male	N
11734	391578	Clearwater	BC	TD Nelson	2018-06-14	51.7100	-120.0334	Adult	Unitrap	Male	N
11735	391579	Clearwater	BC	TD Nelson	2018-06-14	51.7101	-120.0345	Adult	Unitrap	Male	N
11736	391580	Clearwater	BC	TD Nelson	2018-06-14	51.7101	-120.0345	Adult	Unitrap	Male	N
11737	391581	Clearwater	BC	TD Nelson	2018-06-24	51.7100	-120.0334	Adult	Unitrap	Male	N
11738	391582	Clearwater	BC	TD Nelson	2018-06-24	51.7100	-120.0334	Adult	Unitrap	Male	N
11740	391583	Clearwater	BC	TD Nelson	2018-06-24	51.7105	-120.0345	Adult	Unitrap	Male	N
11741	391584	Clearwater	BC	TD Nelson	2018-06-24	51.7105	-120.0345	Adult	Unitrap	Male	N
11743	391585	Clearwater	BC	TD Nelson	2018-07-05	51.7100	-120.0334	Adult	Unitrap	Male	N
11744	391586	Clearwater	BC	TD Nelson	2018-07-05	51.7101	-120.0345	Adult	Unitrap	Male	N
11746	391587	Clearwater	BC	TD Nelson	2018-07-14	51.7100	-120.0334	Adult	Unitrap	Male	N
11747	391588	Clearwater	BC	TD Nelson	2018-07-14	51.7105	-120.0345	Adult	Unitrap	Male	N
11748	391589	Clearwater	BC	TD Nelson	2018-07-14	51.7101	-120.0345	Adult	Unitrap	Male	N
11750	391590	Clearwater	BC	TD Nelson	2018-07-24	51.7105	-120.0345	Adult	Unitrap	Male	N
11751	391591	Clearwater	BC	TD Nelson	2018-07-24	51.7101	-120.0345	Adult	Unitrap	Male	N
11752	391592	Clearwater	BC	TD Nelson	2018-08-03	51.7100	-120.0334	Adult	Unitrap	Male	N
11753	391593	Clearwater	BC	TD Nelson	2018-08-03	51.7105	-120.0345	Adult	Unitrap	Male	N
11754	391594	Clearwater	BC	TD Nelson	2018-08-03	51.7105	-120.0345	Adult	Unitrap	Male	N
11755	391595	Clearwater	BC	TD Nelson	2018-08-13	51.7100	-120.0334	Adult	Unitrap	Male	N
11756	391596	Clearwater	BC	TD Nelson	2018-08-13	51.7105	-120.0345	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
11757	391597	Clearwater	BC	TD Nelson	2018-08-23	51.7100	-120.0334	Adult	Unitrap	Male	N
11758	391598	Crimson Lake PP	AB	TD Nelson	2018-06-22	52.4502	-115.0140	Adult	Unitrap	Male	N
11759	391599	Crimson Lake PP	AB	TD Nelson	2018-07-02	52.4502	-115.0140	Adult	Unitrap	Male	N
11760	391600	Crimson Lake PP	AB	TD Nelson	2018-07-02	52.4506	-115.0148	Adult	Unitrap	Male	N
11761	391601	Crimson Lake PP	AB	TD Nelson	2018-07-02	52.4506	-115.0148	Adult	Unitrap	Male	N
11762	391602	Crimson Lake PP	AB	TD Nelson	2018-07-12	52.4502	-115.0140	Adult	Unitrap	Male	N
11763	391603	Crimson Lake PP	AB	TD Nelson	2018-07-12	52.4502	-115.0140	Adult	Unitrap	Male	N
11764	391604	Crimson Lake PP	AB	TD Nelson	2018-07-12	52.4506	-115.0148	Adult	Unitrap	Male	N
11765	391605	Crimson Lake PP	AB	TD Nelson	2018-07-22	52.4502	-115.0140	Adult	Unitrap	Male	N
11766	391606	Crimson Lake PP	AB	TD Nelson	2018-07-22	52.4504	-115.0141	Adult	Unitrap	Male	N
11767	391607	Crimson Lake PP	AB	TD Nelson	2018-07-22	52.4506	-115.0148	Adult	Unitrap	Male	N
11768	391608	Crimson Lake PP	AB	TD Nelson	2018-08-01	52.4504	-115.0141	Adult	Unitrap	Male	N
11769	391609	Crimson Lake PP	AB	TD Nelson	2018-08-01	52.4504	-115.0141	Adult	Unitrap	Male	N
11770	391610	Crimson Lake PP	AB	TD Nelson	2018-08-11	52.4502	-115.0140	Adult	Unitrap	Male	N
11771	391611	Crimson Lake PP	AB	TD Nelson	2018-08-11	52.4502	-115.0140	Adult	Unitrap	Male	N
11772	391612	Crimson Lake PP	AB	TD Nelson	2018-08-11	52.4502	-115.0140	Adult	Unitrap	Male	N
11773	391613	Red Lodge PP	AB	TD Nelson	2018-08-01	51.9460	-114.2381	Adult	Unitrap	Male	N
11774	391614	Red Lodge PP	AB	TD Nelson	2018-08-01	51.9458	-114.2385	Adult	Unitrap	Male	N
11775	391615	Red Lodge PP	AB	TD Nelson	2018-08-01	51.9457	-114.2390	Adult	Unitrap	Male	N
11776	391616	Red Lodge PP	AB	TD Nelson	2018-08-11	51.9460	-114.2381	Adult	Unitrap	Male	N
11777	391617	Red Lodge PP	AB	TD Nelson	2018-08-11	51.9460	-114.2381	Adult	Unitrap	Male	N
11778	391618	Red Lodge PP	AB	TD Nelson	2018-08-11	51.9458	-114.2385	Adult	Unitrap	Male	N
11779	391619	Red Lodge PP	AB	TD Nelson	2018-08-21	51.9458	-114.2385	Adult	Unitrap	Male	N
11780	391620	Red Lodge PP	AB	TD Nelson	2018-08-21	51.9458	-114.2385	Adult	Unitrap	Male	N
11786	391621	Clearwater	BC	TD Nelson	2018-06-24	51.7105	-120.0345	Adult	Unitrap	Male	N
11788	391622	Clearwater	BC	TD Nelson	2018-07-05	51.7101	-120.0345	Adult	Unitrap	Male	N
11790	391623	Clearwater	BC	TD Nelson	2018-07-24	51.7100	-120.0334	Adult	Unitrap	Male	N
11793	391624	Clearwater	BC	TD Nelson	2018-08-13	51.7105	-120.0345	Adult	Unitrap	Male	N
11800	391625	Clearwater	BC	TD Nelson	2018-06-24	51.7105	-120.0345	Adult	Unitrap	Male	N
12262	391626	Clearwater	BC	TD Nelson	2018-06-24	51.7100	-120.0334	Adult	Unitrap	Male	N
12263	391627	Clearwater	BC	TD Nelson	2018-06-24	51.7100	-120.0334	Adult	Unitrap	Male	N
12264	391628	Clearwater	BC	TD Nelson	2018-06-24	51.7105	-120.0345	Adult	Unitrap	Male	N
12265	391629	Clearwater	BC	TD Nelson	2018-06-24	51.7105	-120.0345	Adult	Unitrap	Male	N
12266	391630	Red Lodge PP	AB	TD Nelson	2018-07-22	51.9460	-114.2381	Adult	Unitrap	Male	N
12267	391631	Red Lodge PP	AB	TD Nelson	2018-07-22	51.9458	-114.2385	Adult	Unitrap	Male	N
12268	391632	Red Lodge PP	AB	TD Nelson	2018-07-22	51.9457	-114.2390	Adult	Unitrap	Male	N
12269	391633	Red Lodge PP	AB	TD Nelson	2018-07-12	51.9457	-114.2390	Adult	Unitrap	Male	N
12270	391634	Red Lodge PP	AB	TD Nelson	2018-07-12	51.9458	-114.2385	Adult	Unitrap	Male	N
12271	391635	Red Lodge PP	AB	TD Nelson	2018-07-12	51.9460	-114.2381	Adult	Unitrap	Male	N
12272	391636	Red Lodge PP	AB	TD Nelson	2018-07-02	51.9458	-114.2385	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
12273	391637	Red Lodge PP	AB	TD Nelson	2018-07-02	51.9457	-114.2390	Adult	Unitrap	Male	N
12274	391638	Red Lodge PP	AB	TD Nelson	2018-07-02	51.9460	-114.2381	Adult	Unitrap	Male	N
12275	391639	Red Lodge PP	AB	TD Nelson	2018-06-22	51.9457	-114.2390	Adult	Unitrap	Male	N
12276	391640	Red Lodge PP	AB	TD Nelson	2018-06-22	51.9458	-114.2385	Adult	Unitrap	Male	N
12277	391641	Red Lodge PP	AB	TD Nelson	2018-06-22	51.9460	-114.2381	Adult	Unitrap	Male	N
12278	391642	Vavenby	BC	TD Nelson	2018-06-24	51.6837	-119.6634	Adult	Unitrap	Male	N
12279	391643	Vavenby	BC	TD Nelson	2018-06-24	51.6844	-119.6633	Adult	Unitrap	Male	N
12280	391644	Near Elk Island NP	AB	TD Nelson	2018-06-21	53.6355	-112.9354	Adult	Unitrap	Male	N
12281	391645	Near Elk Island NP	AB	TD Nelson	2018-06-21	53.6360	-112.9359	Adult	Unitrap	Male	N
12282	391646	Near Elk Island NP	AB	TD Nelson	2018-06-21	53.6355	-112.9369	Adult	Unitrap	Male	N
12283	391647	Near Elk Island NP	AB	TD Nelson	2018-07-01	53.6355	-112.9354	Adult	Unitrap	Male	N
12284	391648	Near Elk Island NP	AB	TD Nelson	2018-07-01	53.6360	-112.9359	Adult	Unitrap	Male	N
12285	391649	Near Elk Island NP	AB	TD Nelson	2018-07-01	53.6355	-112.9369	Adult	Unitrap	Male	N
12286	391650	Near Elk Island NP	AB	TD Nelson	2018-07-11	53.6355	-112.9354	Adult	Unitrap	Male	N
12287	391651	Near Elk Island NP	AB	TD Nelson	2018-07-11	53.6360	-112.9359	Adult	Unitrap	Male	N
12288	391652	Near Elk Island NP	AB	TD Nelson	2018-07-11	53.6355	-112.9369	Adult	Unitrap	Male	N
12289	391653	Near Elk Island NP	AB	TD Nelson	2018-07-21	53.6355	-112.9354	Adult	Unitrap	Male	N
12290	391654	Near Elk Island NP	AB	TD Nelson	2018-07-21	53.6360	-112.9359	Adult	Unitrap	Male	N
12291	-	Near Elk Island NP	AB	TD Nelson	2018-07-31	53.6355	-112.9354	Adult	Unitrap	Male	Y
12292	391655	Near Elk Island NP	AB	TD Nelson	2018-08-10	53.6360	-112.9359	Adult	Unitrap	Male	N
12293	391656	Near Elk Island NP	AB	TD Nelson	2018-08-10	53.6360	-112.9359	Adult	Unitrap	Male	N
12294	391657	Near Elk Island NP	AB	TD Nelson	2018-08-10	53.6355	-112.9369	Adult	Unitrap	Male	N
12295	391658	Red Lodge PP	AB	TD Nelson	2018-07-02	51.9460	-114.2381	Adult	Unitrap	Male	N
12296	391659	Red Lodge PP	AB	TD Nelson	2018-07-02	51.9458	-114.2385	Adult	Unitrap	Male	N
12297	391660	Red Lodge PP	AB	TD Nelson	2018-07-02	51.9457	-114.2390	Adult	Unitrap	Male	N
12298	391661	Red Lodge PP	AB	TD Nelson	2018-07-12	51.9460	-114.2381	Adult	Unitrap	Male	N
12299	391662	Vavenby	BC	TD Nelson	2018-06-24	51.6844	-119.6633	Adult	Unitrap	Male	N
12300	391663	Vavenby	BC	TD Nelson	2018-07-05	51.6844	-119.6633	Adult	Unitrap	Male	N
12301	391664	Vavenby	BC	TD Nelson	2018-07-05	51.6844	-119.6633	Adult	Unitrap	Male	N
12302	391665	Vavenby	BC	TD Nelson	2018-07-14	51.6844	-119.6633	Adult	Unitrap	Male	N
12303	391666	Vavenby	BC	TD Nelson	2018-07-14	51.6845	-119.6627	Adult	Unitrap	Male	N
12304	391667	Vavenby	BC	TD Nelson	2018-08-03	51.6837	-119.6634	Adult	Unitrap	Male	N
12305	391668	Vavenby	BC	TD Nelson	2018-08-03	51.6837	-119.6634	Adult	Unitrap	Male	N
12306	-	Vavenby	BC	TD Nelson	2018-08-03	51.6844	-119.6633	Adult	Unitrap	Male	Y
12307	391669	Vavenby	BC	TD Nelson	2018-08-03	51.6844	-119.6633	Adult	Unitrap	Male	N
12308	391670	Vavenby	BC	TD Nelson	2018-08-03	51.6845	-119.6627	Adult	Unitrap	Male	N
12309	-	Vavenby	BC	TD Nelson	2018-08-03	51.6845	-119.6627	Adult	Unitrap	Male	Y
12310	391671	Vavenby	BC	TD Nelson	2018-07-14	51.6844	-119.6633	Adult	Unitrap	Male	N
12311	391672	Vavenby	BC	TD Nelson	2018-07-24	51.6837	-119.6634	Adult	Unitrap	Male	N
12312	391673	Vavenby	BC	TD Nelson	2018-07-24	51.6844	-119.6633	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
12313	391674	Vavenby	BC	TD Nelson	2018-07-24	51.6845	-119.6627	Adult	Unitrap	Male	N
12314	391675	Vavenby	BC	TD Nelson	2018-08-13	51.6837	-119.6634	Adult	Unitrap	Male	N
12315	391676	Vavenby	BC	TD Nelson	2018-08-13	51.6844	-119.6633	Adult	Unitrap	Male	N
12316	391677	Vavenby	BC	TD Nelson	2018-08-13	51.6844	-119.6633	Adult	Unitrap	Male	N
12317	391678	Nordegg	AB	TD Nelson	2018-08-12	52.4877	-116.0695	Adult	Unitrap	Male	N
12318	391679	Nordegg	AB	TD Nelson	2018-08-12	52.4877	-116.0695	Adult	Unitrap	Male	N
12319	391680	Nordegg	AB	TD Nelson	2018-08-12	52.4877	-116.0695	Adult	Unitrap	Male	N
12320	391681	Nordegg	AB	TD Nelson	2018-08-12	52.4878	-116.0703	Adult	Unitrap	Male	N
12321	391682	Nordegg	AB	TD Nelson	2018-08-12	52.4878	-116.0703	Adult	Unitrap	Male	N
12322	391683	Nordegg	AB	TD Nelson	2018-08-02	52.4877	-116.0695	Adult	Unitrap	Male	N
12323	391684	Nordegg	AB	TD Nelson	2018-08-02	52.4877	-116.0695	Adult	Unitrap	Male	N
12324	391685	Nordegg	AB	TD Nelson	2018-08-02	52.4877	-116.0695	Adult	Unitrap	Male	N
12325	391686	Nordegg	AB	TD Nelson	2018-08-02	52.4878	-116.0703	Adult	Unitrap	Male	N
12326	391687	Nordegg	AB	TD Nelson	2018-08-02	52.4878	-116.0703	Adult	Unitrap	Male	N
12327	391688	Nordegg	AB	TD Nelson	2018-07-23	52.4877	-116.0695	Adult	Unitrap	Male	N
12328	391689	Nordegg	AB	TD Nelson	2018-07-23	52.4878	-116.0703	Adult	Unitrap	Male	N
12329	391690	Nordegg	AB	TD Nelson	2018-07-23	52.4878	-116.0703	Adult	Unitrap	Male	N
12330	391691	Nordegg	AB	TD Nelson	2018-07-13	52.4877	-116.0695	Adult	Unitrap	Male	N
12331	391692	Nordegg	AB	TD Nelson	2018-07-13	52.4878	-116.0703	Adult	Unitrap	Male	N
12332	391693	Nordegg	AB	TD Nelson	2018-07-13	52.4875	-116.0708	Adult	Unitrap	Male	N
12333	391694	Pembina Forks	AB	TD Nelson	2018-07-13	52.9693	-116.6182	Adult	Unitrap	Male	N
12334	391695	Pembina Forks	AB	TD Nelson	2018-07-13	52.9689	-116.6184	Adult	Unitrap	Male	N
12335	391696	Pembina Forks	AB	TD Nelson	2018-07-23	52.9689	-116.6176	Adult	Unitrap	Male	N
12336	391697	Pembina Forks	AB	TD Nelson	2018-07-23	52.9689	-116.6184	Adult	Unitrap	Male	N
12337	391698	Pembina Forks	AB	TD Nelson	2018-07-23	52.9689	-116.6184	Adult	Unitrap	Male	N
12338	391699	Pembina Forks	AB	TD Nelson	2018-08-02	52.9689	-116.6176	Adult	Unitrap	Male	N
12339	391700	Pembina Forks	AB	TD Nelson	2018-08-02	52.9693	-116.6182	Adult	Unitrap	Male	N
12340	391701	Pembina Forks	AB	TD Nelson	2018-08-02	52.9689	-116.6184	Adult	Unitrap	Male	N
12341	391702	Pembina Forks	AB	TD Nelson	2018-08-12	52.9689	-116.6176	Adult	Unitrap	Male	N
12342	391703	Pembina Forks	AB	TD Nelson	2018-08-12	52.9689	-116.6176	Adult	Unitrap	Male	N
12343	391704	Pembina Forks	AB	TD Nelson	2018-08-12	52.9693	-116.6182	Adult	Unitrap	Male	N
12344	391705	Pembina Forks	AB	TD Nelson	2018-08-12	52.9693	-116.6182	Adult	Unitrap	Male	N
12345	391706	Pembina Forks	AB	TD Nelson	2018-08-12	52.9689	-116.6184	Adult	Unitrap	Male	N
12346	391707	Pembina Forks	AB	TD Nelson	2018-08-12	52.9689	-116.6184	Adult	Unitrap	Male	N
12347	391708	Pembina Forks	AB	TD Nelson	2018-08-22	52.9693	-116.6182	Adult	Unitrap	Male	N
12348	391709	Wildhorse Lake PRA	AB	TD Nelson	2018-06-25	53.2719	-117.7909	Adult	Unitrap	Male	N
12349	391710	Wildhorse Lake PRA	AB	TD Nelson	2018-06-25	53.2720	-117.7907	Adult	Unitrap	Male	N
12350	391711	Wildhorse Lake PRA	AB	TD Nelson	2018-06-25	53.2719	-117.7901	Adult	Unitrap	Male	N
12451	391712	Wildhorse Lake PRA	AB	TD Nelson	2018-07-06	53.2719	-117.7909	Adult	Unitrap	Male	N
12452	391713	Wildhorse Lake PRA	AB	TD Nelson	2018-07-06	53.2719	-117.7909	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
12453	391714	Wildhorse Lake PRA	AB	TD Nelson	2018-07-06	53.2719	-117.7909	Adult	Unitrap	Male	N
12454	391715	Wildhorse Lake PRA	AB	TD Nelson	2018-07-15	53.2719	-117.7909	Adult	Unitrap	Male	N
12455	391716	Wildhorse Lake PRA	AB	TD Nelson	2018-07-15	53.2720	-117.7907	Adult	Unitrap	Male	N
12456	391717	Wildhorse Lake PRA	AB	TD Nelson	2018-07-15	53.2719	-117.7901	Adult	Unitrap	Male	N
12457	391718	Wildhorse Lake PRA	AB	TD Nelson	2018-07-25	53.2719	-117.7909	Adult	Unitrap	Male	N
12458	391719	Wildhorse Lake PRA	AB	TD Nelson	2018-07-25	53.2720	-117.7907	Adult	Unitrap	Male	N
12459	391720	Wildhorse Lake PRA	AB	TD Nelson	2018-07-25	53.2719	-117.7901	Adult	Unitrap	Male	N
12460	391721	Wildhorse Lake PRA	AB	TD Nelson	2018-08-04	53.2719	-117.7909	Adult	Unitrap	Male	N
12461	391722	Wildhorse Lake PRA	AB	TD Nelson	2018-08-04	53.2720	-117.7907	Adult	Unitrap	Male	N
12462	391723	Wildhorse Lake PRA	AB	TD Nelson	2018-08-04	53.2719	-117.7901	Adult	Unitrap	Male	N
12463	391724	Wildhorse Lake PRA	AB	TD Nelson	2018-08-14	53.2719	-117.7909	Adult	Unitrap	Male	N
12464	391725	Wildhorse Lake PRA	AB	TD Nelson	2018-08-14	53.2720	-117.7907	Adult	Unitrap	Male	N
12465	391726	Wildhorse Lake PRA	AB	TD Nelson	2018-08-14	53.2719	-117.7901	Adult	Unitrap	Male	N
12466	391727	Obed Lake PP	AB	TD Nelson	2018-06-25	53.5539	-117.1548	Adult	Unitrap	Male	N
12467	391728	Obed Lake PP	AB	TD Nelson	2018-07-06	53.5539	-117.1548	Adult	Unitrap	Male	N
12468	391729	Obed Lake PP	AB	TD Nelson	2018-07-06	53.5539	-117.1548	Adult	Unitrap	Male	N
12469	391730	Obed Lake PP	AB	TD Nelson	2018-07-06	53.5539	-117.1548	Adult	Unitrap	Male	N
12470	391731	Obed Lake PP	AB	TD Nelson	2018-07-15	53.5535	-117.1541	Adult	Unitrap	Male	N
12471	391732	Obed Lake PP	AB	TD Nelson	2018-07-15	53.5536	-117.1546	Adult	Unitrap	Male	N
12472	391733	Obed Lake PP	AB	TD Nelson	2018-07-15	53.5539	-117.1548	Adult	Unitrap	Male	N
12473	391734	Obed Lake PP	AB	TD Nelson	2018-07-25	53.5539	-117.1548	Adult	Unitrap	Male	N
12474	391735	Obed Lake PP	AB	TD Nelson	2018-07-25	53.5539	-117.1548	Adult	Unitrap	Male	N
12475	391736	Obed Lake PP	AB	TD Nelson	2018-07-25	53.5539	-117.1548	Adult	Unitrap	Male	N
12476	391737	Obed Lake PP	AB	TD Nelson	2018-08-04	53.5535	-117.1541	Adult	Unitrap	Male	N
12477	391738	Obed Lake PP	AB	TD Nelson	2018-08-04	53.5536	-117.1546	Adult	Unitrap	Male	N
12478	391739	Obed Lake PP	AB	TD Nelson	2018-08-04	53.5539	-117.1548	Adult	Unitrap	Male	N
12479	391740	Obed Lake PP	AB	TD Nelson	2018-08-14	53.5535	-117.1541	Adult	Unitrap	Male	N
12480	391741	Obed Lake PP	AB	TD Nelson	2018-08-14	53.5536	-117.1546	Adult	Unitrap	Male	N
12481	391742	Obed Lake PP	AB	TD Nelson	2018-08-14	53.5539	-117.1548	Adult	Unitrap	Male	N
12482	391743	McBride	BC	TD Nelson	2018-07-05	53.2864	-120.2003	Adult	Unitrap	Male	N
12483	391744	McBride	BC	TD Nelson	2018-07-05	53.2869	-120.2008	Adult	Unitrap	Male	N
12484	391745	McBride	BC	TD Nelson	2018-07-05	53.2869	-120.2008	Adult	Unitrap	Male	N
12485	391746	McBride	BC	TD Nelson	2018-07-05	53.2869	-120.2014	Adult	Unitrap	Male	N
12486	391747	McBride	BC	TD Nelson	2018-07-15	53.2864	-120.2003	Adult	Unitrap	Male	N
12487	391748	McBride	BC	TD Nelson	2018-07-15	53.2869	-120.2008	Adult	Unitrap	Male	N
12488	391749	McBride	BC	TD Nelson	2018-07-15	53.2869	-120.2014	Adult	Unitrap	Male	N
12489	391750	McBride	BC	TD Nelson	2018-07-25	53.2869	-120.2008	Adult	Unitrap	Male	N
12490	391751	McBride	BC	TD Nelson	2018-07-25	53.2869	-120.2008	Adult	Unitrap	Male	N
12491	391752	McBride	BC	TD Nelson	2018-07-25	53.2869	-120.2008	Adult	Unitrap	Male	N
12492	391753	McBride	BC	TD Nelson	2018-08-04	53.2864	-120.2003	Adult	Unitrap	Male	N

ID	UASM #	Location	Province	Collector	Date Collected	Latitude	Longitude	Life Stage	Method	Sex	Removed
12493	391754	McBride	BC	TD Nelson	2018-08-04	53.2869	-120.2008	Adult	Unitrap	Male	N
12494	391755	McBride	BC	TD Nelson	2018-08-04	53.2869	-120.2014	Adult	Unitrap	Male	N
12495	391756	McBride	BC	TD Nelson	2018-08-14	53.2864	-120.2003	Adult	Unitrap	Male	N
12496	-	McBride	BC	TD Nelson	2018-08-14	53.2869	-120.2008	Adult	Unitrap	Male	Y
12497	391757	McBride	BC	TD Nelson	2018-08-14	53.2869	-120.2008	Adult	Unitrap	Male	N
12498	391758	McBride	BC	TD Nelson	2018-08-24	53.2869	-120.2014	Adult	Unitrap	Male	N
11684	391759	Clearwater	BC	TD Nelson	2017-06-06	-	-	Larva	Hand	Male	N
11711	391760	Vavenby	BC	TD Nelson	2017-07-16	51.6844	-119.6633	Adult	Unitrap	Male	N
11715	391761	Clearwater	BC	TD Nelson	2017-07-17	51.7105	-120.0345	Adult	Unitrap	Male	N
11658 - reseq	-	Clearwater	BC	TD Nelson	2017-07-27	51.7100	-120.0334	Adult	Unitrap	Male	Y
11661 - reseq	-	Clearwater	BC	TD Nelson	2017-07-17	51.7100	-120.0334	Adult	Unitrap	Male	Y
11680 - reseq	-	Clearwater	BC	TD Nelson, BD Wingert, RLK French	2017-07-07	51.7100	-120.0334	Adult	Unitrap	Male	Y
11696 - reseq	-	Clearwater	BC	TD Nelson	2017-06-27	51.7101	-120.0345	Adult	Unitrap	Male	Y

Chapter 3

General Conclusions

Thesis Summary

My primary objective in this thesis was to evaluate the temporal relationship between *Choristoneura fumiferana* and *Choristoneura occidentalis biennis* to explore the mechanisms that help these taxa maintain their genomic integrity.

Spruce budworm species readily hybridize with one another in laboratory settings (e.g. Campbell 1967; Sanders, Daterman, & Ennis 1977; Harvey 1997, Nealis 2005). However, there is some evidence that seasonal temporal isolation may aid in reducing hybridization in natural settings (Smith 1953; Lumley & Sperling 2011), although this is not ubiquitous among all species pairings (Liebhold & Volney 1984; Powell & De Benedictus 1995). Thus, I evaluated the temporal relationship between *C. fumiferana* and *C. o. biennis* across a region of geographic overlap in their ranges to determine if temporal isolation exists between these taxa (Chapter 2). I compared their flight phenology to local degree-day accumulation, making the phenological patterns at different localities more comparable, and in turn allowing insight into whether intrinsic differences between the species, like differential diapause termination, could be the basis for temporal isolation. Additionally, I identified five putative F1 hybrids between these taxa, thereby confirming that their natural hybridization occurs at low rates in the region. I also found *C. o. biennis* east of Edmonton (>100 km further east than previously documented), demonstrating that this species ranges farther from the Rocky Mountains and expected primary hosts than earlier research has indicated. I also identified a few *Choristoneura occidentalis occidentalis* among my collected material, that, despite their clinal relationship (Blackburn *et al.*

2017; Brunet *et al.* 2017) demonstrated that identifiable specimens of both *C. o. occidentalis* and *C. o. biennis* can be present at the same time in some areas of British Columbia. Overall, I found that *C. fumiferana* and *C. o. biennis* have significantly different peaks in their flight period, with collections for 2018 indicating that these peaks differ by as much as four weeks. However, there are still substantial periods during which both species are flying at the same time, suggesting that other mechanisms are reducing their hybridization.

Spruce budworm taxa were identified using genome-wide single nucleotide polymorphisms (SNPs), which provided an opportunity to investigate genomic differences between *C. o. biennis* and *C. o. occidentalis* (Chapter 2). The only consistent phenotypic difference between these taxa is their diapause incidence (Nealis 2005), so we were interested in the function of high-loading SNPs. I evaluated this using BLASTX searches. Two SNPs were associated with heat shock proteins, which may contribute to diapause occurrence. With a completely different dataset, Blackburn *et al.* (2017) also found one heat shock-associated SNP that differed between *C. o. biennis* and *C. o. occidentalis*. Together, these findings provide evidence that SNPs associated with diapause genes are contributing to subspecies-level differences. Additionally, I used the recently published *C. fumiferana* linkage map (Picq *et al*. 2018) to see if these high-loading SNPs were concentrated on any one linkage group (which are approximately equivalent to chromosomes), although many of the scaffolds containing the high-loading SNPs had no match (Chapter 2).

I also attempted collection of *C. o. biennis* using synthetic *C. o. occidentalis* sex pheromones, but was not successful. In 2018, one green Unitrap was baited with 95:5 *E*/*Z*11 tetradecenal flexlures (Synergy Semiochemicals, Burnaby, British Columbia, Canada) and placed near three traps baited with 95:5 *E*/*Z*11 tetradecenal (Sylvar Technologies, Fredericton,

New Brunswick, Canada). I used the protocol of Sanders (1996) to set up the traps. No spruce budworm moths were collected using the lures from Synergy Semiochemicals. Synergy had marketed them as "Western Spruce Budworm" (= *C. o. occidentalis*) lures, which, according to published records of the *C. o. occidentalis* sex pheromone blend (Silk & Kuenen 1988; Silk & Eveleigh 2016), should have been loaded with 92:8 *E*/*Z*11 tetradecenal. After the failure of the traps with these pheromones, I learned that they were loaded with 95:5 *E*/*Z*11 tetradecenal, the ratio intended for *C. fumiferana* (Silk & Eveleigh 2016). More importantly, the Synergy lures had a concentration of 80 ng, three to four orders of magnitude lower (>1000 times weaker) than the 100 μg lures from Sylvar Technologies (D. Wakarchuk, personal communication). Synergy's website now reflects this distinction (https://semiochemical.com/moths-others/). Consequently, no comparisons of sex pheromone differences could be made.

In addition, I attempted to assess *C. fumiferana* and *C. o. biennis* larval association with host foliage in 2017 but was not successful. I used a beating sheet protocol modified from Sweeney, McLean, and Shepherd (1990) to collect larvae at each locality (Chapter 2). My goal was to use these larvae in a comparative study of *C. fumiferana* and *C. o. biennis* development when reared on either white or Engelmann spruce. I collected four spruce budworm larvae across the nine localities, and sequence-based identification was only successful for two of three larvae attempted. Although I did not know the density of spruce budworms prior to larval collection, Unitrap catch of adults indicated that population density was probably too low at many of the localities for successful larval collection (Figure 1, 2017 plots; Rhainds, Therrien, & Morneau 2016). Thus, my low larval yield did not permit any experiments on larval host association and performance.

Future Research

My evidence indicates that seasonal temporal isolation alone is not the primary factor reducing hybridization among these spruce budworm taxa. Although I did not directly test their ability to hybridize in the lab, and I was unable to observe behavioural interactions among the species in the field, there is enough temporal and geographic overlap between *C. fumiferana* and *C. o. biennis* across their range to allow hybridization. Wild-caught hybrids confirm that natural hybridization does occur between these taxa (Brunet 2014; Brunet *et al.* 2017, Chapter 2), but the amount of temporal overlap between them suggests that more hybridization should occur than what I observed. Thus, I recommend the evaluation of other ecological and genetic mechanisms that could reduce hybridization; these are discussed below.

I suspect that hybridization success is reduced when multiple taxa are present in one location. All laboratory tests of hybridization between aldehyde-communicating spruce budworms have used individuals from allopatric populations (Campbell 1967; Sanders *et al.* 1977; Harvey 1997; Nealis 2005). These tests have effectively determined that allopatric populations can hybridize with one another and produce fertile offspring, but they have not determined whether populations of sympatric taxa hybridize with one another. I recommend that "pure" individuals of two taxa should be collected from the same locality, then crossed. Hybridization success could be compared to crosses between the same taxa from allopatric localities. Results of this experiment would elucidate whether hybridization success is lower in areas where the ranges of these species overlap. Bryan Brunet and Giovanny Fagua each attempted such experiments as part of their PhD research in the Sperling lab (F. A. H. Sperling, personal communication), but results were mixed. Few hybrids were produced, but it was unclear whether the crosses failed due to hybrid inviability between the species or mechanical

breakdowns of incubators. Should this experiment be attempted, laboratory conditions must be monitored with the utmost care and numerous replicates should be performed.

Study of spruce budworm sex pheromone blends may reveal subtle differences between species. The full blend of any one taxon has never been determined (Silk & Eveleigh 2016), but each taxon may have minor components in their pheromone blend that help it to distinguish conspecifics from congenerics. Such a relationship would reduce hybridization between taxa that have overlapping populations. Further, pheromone "races" may exist, where different populations within one taxon use somewhat different sex pheromone blends across their range (Emelianov, Drès, Baltensweiler, & Mallet 2001; Bengtsson *et al.* 2014). It may be that reproductive character displacement occurs, i.e. when two spruce budworm taxa are sympatric, sex pheromones traits are under greater differential selection than when allopatric. This could reduce hybridization between taxa in areas where they meet or overlap as they would not recognize one another as ideal mates. To test this, sex pheromones blends of two species and their hybrids should be compared where they are allopatric *versus* where they are sympatric. These blends could be used in field trapping studies in both the allopatric and sympatric ranges of these species. Liebhold, Volney, and Waters (1984) performed a similar study with *Choristoneura retiniana* and *C. o. occidentalis*, although only within the overlapping range of these taxa. They found that hybrid F1 females, hybrid F2 females, and some backcrosses were attractive to males of both parental generations as well as certain hybrid combinations (Liebhold *et al.* 1984). Difficulties arise in collecting enough individuals to test all potential hybrid combinations and backcrosses, so such a study would be complex and likely require experimentation over multiple years.

Host association should also be explored as an isolating mechanism. Populations of each taxon are typically in close association with their primary host, which dictates their distribution (Stehr 1967; Shepherd, Gray, & Harvey 1995). However, various authors have found larval spruce budworms feeding on non-primary hosts (listed in Brown, Robinson, & Powell 2008). It would be informative to see if hybrid F1 larvae are more successful on either primary host of their parental taxa. However, since hybrid F1 larvae can have intermediate development relative to their parents (Volney, Waters, Akers, & Liebhold 1983), synchronizing larvae with host bud flush would be difficult. An intermediate developmental schedule might lead to few adult hybrid F1s, as the larvae may miss the phenological window of either primary host (Lawrence, Mattson, & Haack 1997). However, such an experiment should be designed to avoid confounding effects due to parental stock locality (see above), or introgression among host plant species.

Finally, further work is required to understand the genomic architecture of spruce budworms. My thesis has incorporated a preliminary study of the genomic differences between *C. fumiferana* and *C. o. biennis*, but I found little signal of linkage between markers (although my filtering may have removed any that exists, see Chapter 2). With recent work on linkage groups in the genome of *C. fumiferana* (Brunet 2014; Picq *et al.* 2018), and some potentially adaptive SNPs among spruce budworms (Blackburn *et al.* 2017; Chapter 2), resources are now available to investigate adaptative regions that contribute to the genomic integrity of this species complex. These could involve mechanisms like chromosomal inversions that stop adaptive genes from recombination, and thereby contribute to speciation of the spruce budworms, although this still must be tested. One finding that suggests blocks of co-adaptive genes is that many traits that differ between spruce budworm species are associated with or restricted to the X chromosome (Sperling 1994).

In conclusion, understanding how temporal isolation contributes to speciation and diversity within spruce budworms is of ecological and economic importance. Climate warming has recently been implicated in phenological change of *C. fumiferana* (Pureswaran, Neau, Marchand, De GrandPré, & Kneeshaw 2019), and feeding by this species and other spruce budworms can cause serious forest losses (Volney & Fleming 2007). Should the developmental characteristics of spruce budworm taxa undergo major change, atypical host conifers may be at risk of severe defoliation. This thesis contributes to the already vast body of research on the spruce budworm species complex and furthers our understanding of their interactions in natural environments. Future explorations of spruce budworm ecology and evolution will be important to understanding how each taxon maintains its genomic integrity, and such studies will also shed new light on the process of ecological speciation.

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Biography

I was born on 16 October 1992 at the Colchester East Hants Health Centre in Truro, Nova Scotia. As the first of two children of my parents, Larry Nelson and Laurel Lea Nelson (née Stewart), I played perhaps the typical role of older brother to my sister, Hayley. We lived in Fletchers Lake, Nova Scotia, where I recall spending great portions of time exploring our "backyard" – the seemingly endless woods of the neighborhood. I collected frog eggs from the brook that ran through these woods, and "skated" (walked the ice) on its ponds.

My entomological experiences began around this time. My first encounters with insects involved the collection of what I called "grass moths" from the lawn of our yard (crambids, likely) and cicadas landing on laundry that hung from the clothesline. We also had the odd luna moth that would come to the light outside of our basement door. During a Christmas break while finishing my Masters, my dad told me about his attempt to get me into baseball when I was about five years old, where I spent my outfield time looking at bugs on the ground. He had a good-natured laugh as he told me, and I am sure he was just as thrilled with my sportsmanship in 1997.

My athleticism did improve, however, as I later became a sprint kayaker at Cheema Aquatic club. I also worked as a lifeguard at this club during the summers. These "pastimes" consumed nearly all my time throughout junior high and high school, but I left the sport soon before enrolling in an undergraduate program at Acadia University in 2011. During that degree, I participated in various extracurricular activities: becoming station director of the campus radio, copy editor of the school newspaper, and playing in a few bands (including a Weezer cover band, "Coff"). Most notably, though, I joined the co-operative education program that allowed me to work an eight-month contract at the Kentville Research and Development Centre as an entomological research technician. I enjoyed the experience so much that I sought out a similar

position at the Atlantic Forestry Centre, which led to an undergraduate honours thesis on brown spruce longhorn beetle, an invasive forest pest in the maritime provinces of Canada.

All of this led to graduate school in 2016. Initially, I had intended to study native Albertan bees with Dr. Jessamyn Manson at the University of Alberta. Through a series of odd circumstances, I instead ended up studying the spruce budworm species complex in Dr. Felix Sperling's lab. In hindsight, this was certainly a fortunate turn of events. Although I knew very little in the grand scheme of things (and still know very little), I was probably best suited to continue working with forest pests. My time in the Sperling lab has been very valuable; I feel I have learned a great deal about molecular biology while still getting to spend a lot of time collecting insects in the field. And of course, I have met many like-minded bug nerds who have further contributed to my interest in the charismatic microfauna below our feet.

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