# Cryptic Host-Associated and Frequency-Dependent Patterns of Host Species Selection of a Candidate Weed Biological Control Agent in its Native Range

by

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## ABSTRACT

At least nine species of European hawkweeds in the genus *Pilosella* Vaill. (Asteraceae) are invasive in western North America, where they are a detriment to natural and managed lands and have vast economic impacts. A promising candidate biological control (biocontrol) agent for multiple species of *Pilosella* is the gall inducing wasp *Aulacidea pilosellae* Kieffer (Hymenoptera: Cynipidae). Initial studies of this candidate agent revealed the potential for two biotypes of the insect that differed in physiological host range, voltinism, and reproductive mode. It was hypothesized that these differences were related to geographic separation of the populations and it was further observed, within one subset of the distribution, that different host species were being used at nearby sites, despite similar host species being present. The overarching goal of this thesis was to increase the understanding of the patterns of host species use by *A. pilosellae* in order to inform the biocontrol programme for invasive *Pilosella* hawkweeds. The specific objectives were to (i) test the hypothesis that *A. pilosellae* has definitive preferences for species of *Pilosella* within its ecological host range, (ii) further quantify the ecological host range of *A. pilosellae* by conducting systematic surveys across a broad geographic distribution, and (iii) test the hypothesis that differences between the purported biotypes were due to cryptic genetic differentiation, predicted to be based primarily on geographic location of populations and secondly on host-association. By utilizing an integrative approach of thorough and widespread field surveys and molecular methodologies, this thesis presents findings that substantially increase the understanding of patterns of host species use by *A. pilosellae* in its native range. Specifically, (i) the hypothesis of host preferences of *A. pilosellae* is rejected in favor of frequency-dependent host species selection, (ii) five species of *Pilosella* invasive in North America are confirmed as hosts of *A. pilosellae* in its native range and (iii) the hypothesis that cryptic genetic differentiation exists within the species *A. pilosellae* was supported. However, the prediction that differentiation was based primarily on geographic separation was rejected in favor of differentiation based on a combination of disjunct host-associations, infection with the bacterial endosymbiont *Wolbachia*, and to a lesser extent, on geography.

# PREFACE

With guidance from my co-supervisors Jason Pither and Bob Lalonde, my committee members Rose De Clerck-Floate and John Klironomos, and collaborator Gitta Grosskopf-Lachat, I was responsible for the design, implementation and analysis of the research presented in Chapter 2: Quantifying patterns of host plant species use by *Aulacidea pilosellae*, a candidate weed biological control agent for *Pilosella* hawkweeds, in its native range. I was assisted in this aspect of the work by field assistants Dave Ensing and Jeff Padgham.

With guidance from my co-supervisors Jason Pither and Bob Lalonde, my committee members Rose De Clerck-Floate and John Klironomos, and collaborator Kevin Floate, I was responsible for the design and analysis of the research presented in Chapter 3: Cryptic diversity of a candidate weed biocontrol agent – Due to geography, host-association or *Wolbachia* infection? The implementation of the molecular work (DNA extraction and PCR amplification) was primarily conducted in the lab of Kevin Floate by technician Paul Coghlin, although I performed initial DNA extractions. Paul Coghlin also sent the PCR product for sequencing, performed the initial BLASTn which revealed the CO1 sequence JN288739, and provided the raw sequence data to me, which I analyzed with advice from and modified analysis code obtained from Michael Russello.

I was responsible for the writing of this thesis in its entirety, with helpful suggestions and editing that improved the thesis from my co-supervisors.

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*To those that have enabled me to have the privilege*

*and for those that are not afforded the same opportunity*

# CHAPTER 1: GENERAL INTRODUCTION

## INTRODUCTION

Invasive species are one of the greatest threats to global biodiversity (Simberloff 2000), and can have dramatic impacts on ecosystem structure and function (Mack et al. 2000). Economic losses, due to the direct and indirect impacts of invasive species on resource based activities, as well as efforts to control invasive species and mitigate their impacts, are estimated at $1.4 trillion USD annually (Burgiel and Muir 2010). While control programmes of all types can vary in their success, biological control is among the most important and effective tools used to control invasive species (McFadyen 1998).

### Biological control of invasive plant species

Biological control can be defined as "the actions of parasites, predators, and pathogens in maintaining another organism's density at a lower average than would occur in their absence" (DeBach 1964). These parasites, predators or pathogens are commonly referred to as biological control (biocontrol) agents. Biological control can be further divided into three types: conservation – encouragement of existing biocontrol agents, augmentation – intervention to increase agent populations, or classical – the intentional importation and release of specialized natural enemies to control exotic species (McFadyen 1998). Classical biological control, which aims to permanently establish agents without the need for augmentation, is the most common type of biocontrol utilized in the management of invasive plant species (McFadyen 1998). When successful, classical biological control leads to long-term, sustainable control of invasive plant populations, reducing or eliminating the need for chemical control, and is ultimately cost effective (Hoddle 2004). While most biological control programmes indeed require a heavy financial investment, estimated at between half a million to one and a half million dollars per programme (R. De Clerck-Floate, *pers. comm.*), the success of such programmes has not been frequently evaluated, thus estimates on the return for investment are few. Where such calculations have been made is generally in successful systems: calculations for the biological control of knapweed in British Columbia estimate the benefit:cost ratio were 17:1, whereas a chemical control programme likely would have yielded a benefit:cost between 0.78 and 1.05:1 (Frid et al. 2009).

Carson (1962) credits physician Erasmus Darwin (grandfather of Charles Darwin), with being the first to suggest that a given species (an insect) may be controlled by encouraging its natural enemies. While research in the field of biological control, especially against arthropod agricultural pests, was fairly common in the first half of the 20th century, research in the field burgeoned after the publication of Carson's *Silent Spring* (Barratt et al. 2010). The first use of a biological control agent for a weed was the release of the cochineal (a scale insect) on *Opuntia* in India in 1863 (Tryon 1910). In the early history of classical biological control, candidate agents were not formally tested as they are today and instead emphasis was placed on observations made when initially collecting the insects in the country of origin (Briese and Walker 2002). By the 1920s, the first formal host-specificity tests were conducted using economically important cultivated crops (Briese and Walker 2002). As early as the mid-1960s, scientists involved in weed biocontrol recognized the need for the investigation of not just crop species, but also the "biology, specificity and evolutionary relationships between the insect and its host plant" (Harris and Zwölfer 1968). Building upon the criteria proposed by Harris and Zwölfer (1968), Wapshere (1974) outlined the basis of host plant specificity testing today, known as the *Centrifugal Phylogenetic Method*. The *Centrifugal Phylogenetic Method* intends to define the host range (the list of species used as hosts, Bernays and Chapman 1994) of candidate agents and minimize negative impacts on economically important species. Wapshere (1974) describes this *Centrifugal Phylogenetic Method* as applying to those candidate agents already deemed "sufficiently specific and sufficiently damaging" i.e. the method does not apply to the majority of (polyphagous) organisms associated with the targeted weed, which should be pre-excluded.

Following the *Centrifugal Phylogenetic Method*, once candidate agents are selected and obtained, a candidate agent is first exposed to test plants most closely related to the target weed (those in the same genus or sub-genus), and then more and more distant relatives of the target weed until the host range is defined (Wapshere 1974). An updated approach, proposed by Briese (2005) tests plant species based on phylogenetics relatedness to the target weed, and additional plant species are selected for testing if they share plant chemistry with the target weed. Under current testing regimes, economically important crop species in the same family as the target weed are still tested, and particular attention is paid to rare and endangered related native plant species and to host plants of congeners of the candidate agent (Schaffner 2001). This modified *Centrifugal Phylogenetic Method* of host range testing is designed to test the physiological (syn. fundamental) host range of an insect, that is the set of species on which an insect can complete

development under no choice conditions (Schaffner 2001). This is a logical and necessary first step in risk assessment: testing the physiological host range is essential to determine potential threats to native, endangered and economically important species. A small number of introductions of agents, targeting arthropods or weeds, have had adverse consequences on non-target species and have led to the publication of many papers cautioning against the use of biological control (e.g. Louda et al. 1997, Simberloff and Stiling 1996). Termed the "*Achilles' Heel of biological control*" by Louda et al. (2003), ecological risk to non-target species drives heated debate about the safety of biological control. However, the physiological host range is much broader than the realized or ecological host range; the set of plant species actually utilized by the insect under natural conditions (Schaffner, 2001).

While focusing attention on determining the total set of plant species that may be acceptable hosts under dire conditions is effective in providing a robust list of potential host plants, testing of the physiological host range alone does not provide sufficient information to predict which of these hosts will actually be utilized. Of thirty candidate weed biocontrol agents tested at CABI Europe-Switzerland up until 2001, only one proved to be truly monophagous under no-choice conditions, and 24 of the 30 had a few individuals that were able to develop on hosts not found to be utilized by the insect in extensive field surveys (Schaffner 2001). Low levels of non-target attack on closely related species have not historically precluded agents from introduction (Palmer 2004) as it is recognized that incidental feeding under artificial conditions likely grossly overestimates the ecological host range. Thus, if agents were to be assessed on the basis of physiological host range testing alone and if any non-target feeding were deemed unacceptable, the vast majority of potentially successful candidate agents would be eliminated and few options for control would remain. Subsequent to testing of the physiological host range, current testing includes assessing the ecological host range of a candidate agent in a multiple-choice setting, using any plant species identified as physiological hosts in the no-choice testing, along with the target weed. It should be further noted that in order for a test plant species to be considered within the physiological host range of the candidate agent, that agent must complete development on that test plant species.

The current climate of high government risk-aversion to biological control presents new challenges. Candidate agents that have demonstrably high control efficacy on the target plants may also be those that have the highest potential for non-target effects. Indeed, several of

Canada's most 'successful' weed biological control agents would likely not be released under today's strict standards. One of Canada's most successful weed biocontrol agents, the weevil *Rhinocyllus conicus* Froel., was released to control the invasive milk-thistle, *Carduus nutans* (Zwölfer and Harris 1984). Subsequent to its introduction, *R. conicus* received immense negative attention in the literature due to its taste for several native North American thistles (Louda et al. 1997), in particular a threatened and endangered thistle species in the United States. While authors such as Louda et al. (1997) aptly highlight the negative consequences of this agent, the public perception that the *R. conicus* story is one of releasing an insect presumed to be host-specific that then expanded its host range is untrue – and in fact, this phenomenon has not been documented to occur for any weed biocontrol agent. During host specificity testing it was apparent that *R. conicus* fed on thistles in at least four genera, but at rates much lower than on *C. nutans* (Zwölfer and Harris 1984). While the risk to native thistles such as the threatened and endangered US species was documented, the social acceptance of such risk has changed since this introduction (R. De Clerck-Floate, *pers. comm.*), spurring the critiques of the *R. conicus* release by Louda et al. (1997) and others. More recently, another weevil, *Mogulones crucifer* Pallas was released in western Canada in 1997 to control invasive houndstongue, *Cynoglossum officinale*, and has also been a highly successful agent (De Clerck-Floate and Wikeem 2009). However, *M. crucife*r has also shown low-levels of non-target attack in the introduced range on certain members of the related species in the family Boraginaceae, including *Hackelia* spp. (Andreas et al. 2008), of which one species in the United States is considered threatened and endangered. As such, the transportation and release of *M. crucifer* in the United States is considered a criminal offense under the US Endangered Species Act (USDA-APHIS 2010, available at http://oregonstate.edu/dept/nurspest/Mogulones_Cruciger.pdf). This agent would certainly not be released in the current climate of high risk aversion. More recently, a promising candidate agent (*Aceria solstitialis*) for yellow starthistle (*Centaurea solstitialis*) was petitioned for release in the United States, but was rejected as it showed (very) limited attack on safflower and cornflower, two economically valuable species, under no-choice conditions (L. Smith, *pers. comm.*). Stoeva et al. (2012) conducted extensive testing to demonstrate that non-target attack by *A. solstitialis* only occurred under no-choice conditions, and not in any multiple-choice tests or even when *A. solstitialis* was released on safflower fields in the native range, yet the petition for release was still rejected due to the perceived (but scientifically unsupported) risk to economically important species. Current risk averse policies thus severely limit weed biocontrol

scientists by eliminating the majority of candidate agents. The agents that remain are likely those that do not have the highest impact on the target plant.

It should be noted that laboratory and native range test conditions may not reflect how much of an effect an agent will have once released in an invasive population. Many agents are thought to have more adverse impacts in the introduced rather than the native range, due at least in part to a phenomenon known as the evolution of increased competitive ability, a hypothesis proposed by Blossey and Notzold (1995). They observed that not only did plants of the same species grown in the invaded range produce more biomass than plants in the native range, but that an herbivorous insect (used as a biocontrol agent) experienced higher performance on the plants from the introduced range. Other reasons proposed for increased success of weed biocontrol agents in the introduced range includes the enemy release hypothesis – a hypothesis also proposed to explain why invasive plants are more successful in the introduced range - which provides the theoretical rationale for biological control (Elton 1958, Keane and Crawley 2002). According to the enemy release hypothesis, biological control agents may have a higher impact in the absence of their own specialized enemies (Hawkins et al. 1993, Hawkins and Cornell 1994). However, to what extent a release in parasitoid load may improve an agent's ability to control its target weed or the degree to which novel parasitoids may attack the agent in the introduced range could be very difficult to predict (McFadyen and Spafford Jacob 2004, McClay and Balciunas 2005).

Factors in addition to agent safety should be considered in biological control programmes. Considerations such as agent efficacy on target weed individuals and modelling of predicted impacts to the target weed at the population level, as well as climatic matching of the distribution of the weed and the physiological requirements of the insect have become important components of weed biological programmes (McClay and Balciunas 2005). A unique challenge is posed by weed biological programmes that target complexes of invasive congeners. Deciding whether to target all congeners or a select few that pose the greatest risk is difficult, and especially difficult if the target species are morphologically challenging to discern from each other. Finding an agent that may attack multiple congeners is a relatively easy task, but predicting which congeners will likely be controlled by such an agent is more difficult. This would require not only no-choice and choice experiments to determine physiological host preferences, but ideally extensive surveys across the broad native range distribution of the candidate agent, in order to

quantify observed preference and patterns of ecological host use. Thus, defining the ecological host range in native range communities could be of great predictive power for controlling complexes of closely related invasive species.

A number of papers in the field of weed biocontrol articulate a need for increased native range investigations. Many of these papers (e.g. Hinz and Schwarzlaender 2004, Hierro et al. 2005, Cripps et al. 2006) suggest comparative studies of plant demography, ecology or herbivory of invasive species in both the native and invaded ranges in order to better understand weed and control agent dynamics. Others, such as Jongejans et al. (2006) argue that a first step in biological control should be investigating what factors regulate the population dynamics of the target plant species in its native range. Schaffner (2001) reasons that potentially (biocontrol) programme-altering information could be gathered by conducting surveys of sympatrically occurring, related plant species, in order to assess the realized or ecological host range of candidate agents in their native environment. More broadly, Goolsby et al. (2006) argue that "[n]ative range-work ... offers under-exploited opportunities for contributing towards predicting safety, abundance and efficacy of potential agents in their target environment".

**Application of molecular methods to weed biological control**

Further focus in recent years has been placed on the application of molecular methodologies to selecting candidate agents for weed biological control (Goolsby et al. 2006, Gaskin et al. 2011). A number of molecular methods (reviewed in Gaskin et al. 2011) can be employed to aid in clarifying both the taxonomic and evolutionary relationships of weeds and their candidate agents, discovering cryptic taxa, determining population genetic structure, locating centres of origin of weeds in order to maximize potential agent species, and identifying immature life stages of candidate agents. The method employed can depend on many factors, including expertise of the research team, the specific research question, how much prior information is available on the taxa of interest, and budgetary/time constraints (Gaskin et al. 2011).

DNA sequencing is now a widely established method of investigating cryptic diversity and host specificity of many insect taxa (Hebert et al. 2004), including some arthropod biological control agents (reviewed in Gariepy et al. 2007). In insects, the most common gene regions targeted for sequencing are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) (Gariepy et al. 2007). The mtDNA cytochrome oxidase I (COI) subunit is by far the most widely used, due

to its promotion as the 'DNA barcode' region and its ability to delimit species across animal taxa (e.g. Hebert et al. 2003). The rDNA internal transcribed spacer I and II (ITSI and ITSII), and 18S and 28S D2 rDNA subunits are among the most widely used rDNA regions in insects, as these regions also show considerable variation in many taxa at the genus and species levels (Campbell et al. 1993, Rokas et al. 2002b, Li et al. 2010), but often less variation than in mitochondrial regions (Avise 1994). While there are many opponents to DNA barcoding who argue against its use in taxonomy all together (e.g. Will et al. 2005, Song et al. 2008), others take a more moderate approach and urge the use of multiple markers (e.g. Rubinoff and Holland 2005). Specifically, using both mitochondrial and nuclear gene regions in DNA sequencing studies has been suggested, as it has been demonstrated in a number of cases that mtDNA sequences can yield different phylogenies than nuclear DNA sequences from the same organisms (Ballard and Whitlock 2004, Shaw 2002). Others still demonstrate that iterative methods of combining DNA sequence data with morphological and ecological data (e.g. Smith et al. 2008, Tan et al. 2010) yield phylogenies that better estimate true evolutionary relationships among taxa. More recent studies commonly follow these suggestions, and employ both multiple markers and ecological and/or morphological data.

Only recently applied to the biological control of weeds, DNA sequencing can be a powerful component of resolving host-associations, phylogenies and evolutionary relationships, especially when combined with ecological and/or morphological data. Toševski et al. (2011) investigated the potential for cryptic variation in the successful weed biocontrol agent of Dalmatian toadflax (*Linaria dalmatica*), the weevil *Mecinus janthinus* Germar. Three species of toadflax, *L. dalmatica, L. genistifolia,* and *L. vulgaris*, are all weeds of European origin that have invaded North America. Taxonomy of the genus *Linaria* is yet unresolved, especially the *L. dalmatica/genistifolia* complex of species and hybrids (Toševski et al. 2011). The majority of *M. janthinus* released in North America were collected from *L. vulgaris* in Europe, and a small population collected from a subspecies of *L. dalmatica* (Toševski et al. 2011). However, in North America the agent was successful on *L. dalmatica* and only rarely found on *L. vulgaris*. This led Toševski et al. (2011) to speculate whether there was cryptic host-associated genetic diversity of *M. janthinus*. Using DNA sequencing of mtDNA COII, combined with a host-suitability study and morphological examination of specimens, they revealed there are in fact at least two cryptic host-associated species that have been recognized as *M. janthinus*.

Another recent study (Szűcs et al. 2011) investigated establishment success of two known biotypes of the flea beetle *Longitarsus jacobaeae* Waterhouse, which has successfully controlled *Jacobaea vulgaris* (tansy ragwort) in western North America. The two biotypes, which cannot be distinguished morphologically, were released in 1969: one from Italy, which is presumed to have been the successful type, and one cold-adapted type from Switzerland, which was re-released in 2002 and again in 2011 (R. De Clerck-Floate, *pers. comm.*). Using the technique of Amplified Fragment Length Polymorphisms (AFLPs), Szűcs et al. (2011) were able to determine which biotypes had established at particular sites in their study area, and detect hybridization events.

While both Toševski et al. (2011) and Szűcs et al. (2011) utilized molecular methods to address questions related to the post-release success of weed biological control agents, molecular methods have even greater potential for addressing questions of host-specificity in pre-release studies of candidate weed biocontrol agents. Studies that utilize molecular methods to assess the host range and the existence of cryptic diversity of candidate weed biocontrol agents in pre-release testing are becoming more common. Rector et al. (2010) used DNA sequencing to assess host-use by the weevil *Ceratapion basicorne* Illiger, a candidate bio-agent for *Centaurea solstitialis* (yellow starthistle), and to identify other herbivores associated with *C. solstitalis*. Rauth et al. (2011) examined the population structure of the weevil *Ceutorhynchus scrobicollis* Nerensheimer and Wagner, a candidate biocontrol agent for *Allaria petiolata* (garlic mustard), using both DNA sequencing of mtDNA and AFLPs, and found that the sites from which *C. scrobicollis* had been collected represented a connected meta-population, and determined that individuals used in the host-specificity testing represented enough genetic diversity to accurately assess the host-range. This increase in the utilization of molecular tools in pre-release studies is likely due to a general increase in knowledge and awareness among weed biocontrol scientists regarding the molecular tools available and how these tools can be utilized to provide more detailed host-specificity information. It is therefore becoming widely accepted that utilizing molecular tools can be a critical step in pre-release agent assessment, as the use of specialized lineages or biotypes, if they are found to exist, may increase the efficacy and safety of weed biocontrol programmes (Goolsby et al. 2006, Gaskin et al. 2011).

Despite its many potential benefits, the use of molecular methods such as DNA sequencing to assess the safety and efficacy of candidate agents is not without limitations. It is widely

recognized that such molecular investigations should be complemented by ecological investigations. Further emphasis is placed on the comparison of morphological characters if genetic differences are found. The latter are likely more applicable to studies that attempt to clarify the taxonomic status and describe new species where cryptic diversity is found.

### *Wolbachia* infection and potential impacts on its hosts

Another limitation that may affect the interpretation of the results of mtDNA sequence data in particular, is the potential for insects to be infected with bacterial endosymbionts, such as *Wolbachia*. *Wolbachia* is a genus of obligate intracellular alphaproteobacteria known to infect a significant proportion of arthropods. *Wolbachia* have a range of phenotypic effects on their hosts, and generally act as reproductive parasites, altering the biology of the host to the benefit of the endosymbiont (Werren et al. 2008, Hornett et al. 2010). While many other types of endosymbiotic bacteria infect insects with similar results (e.g. *Cardinium* and *Rickettsia)*, much less research has been conducted on these other groups, and they are not discussed further.

Many studies state that about 20% of insect species are infected with *Wolbachia*, however, a recent meta-analysis estimates that more than 65% of insect species carry *Wolbachia*, and that at least $10^6$ insect species are infected (Hilgenboecker et al. 2008). *Wolbachia* generally spread within species vertically, through maternal transfer in egg cytoplasm, but are also known to spread horizontally (Frydman et al. 2006).

*Wolbachia* are known to induce four types of reproductive manipulations in their hosts; (i) feminization (genetic males that develop as females), (ii) male killing (the death of infected males during embryogenesis, resulting in more available resources for infected female siblings), (iii) cytoplasmic incompatability (CI, the prevention of infected males from mating with females that do not share the same *Wolbachia* type) and (iv) induced parthenogenesis (the production of daughter offspring from unfertilized eggs, due to embryonic disruption that yields diploidy), the latter two of which are induced by *Wolbachia* in the hymenoptera (Werren et al. 2008).

*Wolbachia* infections have been linked to cryptic host-associations in insects (Sun et al. 2011) and may affect the diversity of mtDNA present within taxa (Hurst and Jiggins 2005). *Wolbachia* infections are of further interest to biological control, as *Wolbachia* have the ability to induce a

number of reproductive alterations in their hosts, which may be of benefit or detriment to a biological control programme (Floate et al. 2006).

*Wolbachia* infections are prolific in the Cynipidae, a family of Hymenopteran gall inducers, and increasing evidence suggests *Wolbachia* infections may play a large role in the varying reproductive modes observed in this group. Among the six tribes of Cynipids, the two oak-galling tribes Pediaspidini and Cynipini both reproduce via cyclical parthenogenesis. The remaining rose-galling tribe Diplolepidini, acacia-galling tribe Eschatocerini, paraphyletic herb-galling tribe the 'Aylacini' (inverted commas are used due to paraphyly, after Liljeblad and Ronquist 1998) and inquiline (an animal that lives commensally in the dwelling of another animal, in this case the gall made by another gall-inducer) tribe Synergini, which has lost its ability to induce galls itself, are all known to reproduce via arrhentoky. Arrhentoky occurs whereby unfertilized eggs produce haploid males and fertilized eggs produce diploid females. Despite this broad pattern, many species in these gall-inducing-tribes are only known from thelytokous (parthenogenesis in which females are produced from unfertilized eggs) generations (Rokas et al. 2002a). In all such populations/species of galling Cynipids that have been tested for *Wolbachia*, there has been a positive association between thelytoky and *Wolbachia* infection (e.g. Plantard et al. 1998, 1999).

*Wolbachia* infections in other groups of hymenoptera have also been associated with thelytoky. In several well studied examples, infected females treated with antibiotics reverted to arrhentoky, e.g. *Trichogramma* spp. (Stouthamer et al. 1990), and *Muscidifurax* spp. (Stouthamer et al. 1993) but see Plantard et al. (1998) for a good synopsis. Many species of Cynipid rose- and herb-gallers are considered thelytokous due to the scarcity of males (Eady and Quinlan 1963, Nieves-Aldrey 1980, Askew 1984). When the reproductive mode of ten populations of the rose-galler *Diplolepis spinosissimae* was investigated, eight populations were found to be thelytokous (with an cumulative frequency of 1.3% males in the combined populations) and also infected with *Wolbachia*; while the remaining two populations were found to be arrhentokous (with males representing 21 and 29% of those populations) and not infected with *Wolbachia* (Plantard et al. 1998a). The authors reason that the occurrence of the few males (and a few un-infected females) in these thelytokous/*Wolbachia* positive populations are due to loss of *Wolbachia*, possibly through incomplete maternal transmission, environmental curing agents, or prolonged larval diapause (Plantard et al. 1998). A further study tested for *Wolbachia* infection in the Rhoditini

(synonym for the Diplolepini or rose-gallers) and the 'Aylacini,' and found *Wolbachia* infections in twelve of the nineteen Rhoditi species tested and in four of the eight 'Aylacini' species tested (Plantard et al. 1999). Of the eight species of Aylacines tested, the reproductive mode of six had been inferred by Folliot (1964) based on offspring sex ratios of virgin females. All of the four species that tested negative for *Wolbachia* were previously described as arrhenotokous by Folliot, one such species being *Aulacidea hieracii* Bouché, a gall inducer on species of true *Hieracium*. Of the four species that tested positive, Folliot had described the reproductive mode of two, both of which were thelytokous. Thus, there is widespread direct and indirect evidence that *Wolbachia* induce thelytokoy in normally arrhentokous Cynipids and other hymenopterans.

Indicative of their vertical/maternal transfer into the host genome, *Wolbachia* infections can have strong associations with certain mitochondrial genotypes (Werren et al. 2008). Such associations between *Wolbachia* infection and mtDNA genotypes could arise due to the proliferation of infected females resulting from the reproductive manipulations induced by *Wolbachia* in their hosts. Hurst and Jiggins (2005) argue that this form of strong association between *Wolbachia* and mtNDA genotypes makes using mtDNA in phylogenetic studies of infected species potentially unreliable. The basis of these concerns lies in the potential for *Wolbachia* to drive non-neutral selection of mtDNA in their hosts, by either reducing or increasing mtDNA variation in a species. Such an increase in mtDNA diversity could occur as a result of *Wolbachia* infection separating mtDNA lineages to such an extent that multiple species are hypothesized when in fact only one exists (Smith et al. 2012). A decrease in mtDNA diversity could thus result due to a *Wolbachia* induced 'selective sweep' – in effect, an elimination of mtDNA variation via hybridization events that result in the fixation of the endosymbiont (Smith et al. 2012).

Smith et al. (2012) specifically examine how common the incidence of *Wolbachia* conflating mtDNA events actually are, and found very low incidences of mito-nuclear sequence discordance associated with *Wolbachia* infection. They also compile a few studies across insect taxa (Diptera, Hymenoptera, Lepidoptera) where patterns of interspecific mitochondrial introgression have been found in sister species and where *Wolbachia* was also present, and has been hypothesized to play a causative role in such events. The extent to which these types of alterations in mtDNA is caused by *Wolbachia* remains largely unresolved; however, the possibility that *Wolbachia* infection may confound mtDNA presents further reason to utilize not

only mitochondrial gene regions in DNA sequencing studies. Further, Floate et al. (2006) highlights the importance of testing for *Wolbachia* infection in candidate biological control agents. The reproductive manipulations induced by *Wolbachia* can result in dramatic population level alterations, including adaptive changes to offspring sex ratio, and impacts on fecundity in infected populations. Thus, *Wolbachia* infection has the potential to be of either benefit or detriment to biological control programmes and screening of candidate agents should become routine.

## STUDY SYSTEM

### Invasive hawkweeds

There are at least nine species of the genus *Pilosella* Vaill. (Asteraceae: Lactuceae) that have been introduced into western North America (Table 1.1) and more species still present in eastern provinces and states (Wilson 2006, revised 2007). The genus *Pilosella*, also commonly known in North America by its synonym *Hieracium* subgenus *Pilosella*, is recognized as a distinct genus in its native Europe (Tyler 2001, Bräutigam and Greuter 2007), and thus is only represented by non-native species in North America. All hawkweed species native to North America are members of the genus *Hieracium*. While there are also *Hieracium* species of European origin that are invasive in North America, these species are not targeted for biological control thus are not considered further.

Species of invasive *Pilosella* vary widely in their distributions and impacts. Some species are widely distributed across the western provinces and states (e.g., *P. caespitosa* and *P. aurantiaca*) while others are merely known from a single occurrence, (e.g., *P. lactucella*, Wilson 2006, revised 2007). The actual distributions of most of these species are not well quantified. All species of invasive *Pilosella* are yellow flowering with the exception of *P. aurantiaca*, and are commonly not identified to species level but recorded simply as ‘hawkweed species’ or ‘yellow hawkweed’ in invasive species databases. However, current efforts are underway to resolve species-specific distributions. Further complicating identification and thus accurate assessments of species-specific impacts, is that the *Pilosella* hawkweeds are characterized by apomixis, polyploidy and frequent hybridizations, which yields a complex of morphologically similar forms (Fehrer et al. 2005) even in the invaded range.

As a genus, invasive hawkweeds pose serious economic and environmental threats in western North America (Wilson et al. 2006). Invasive hawkweeds were likely first introduced into North America in the early 19th century. Since introduction, these species have established as aggressive invaders that out-compete crops and forage vegetation in fields and pastures and replace native vegetation in undisturbed natural areas, threatening biodiversity and rare species (Lass and Callihan 1997). These highly competitive weeds are primarily managed by herbicides in areas of economic importance (Wilson et al. 2006) which have their own environmental, animal and human health impacts. In the vast natural areas hawkweeds invade they continue to spread in the absence of natural enemies. Within the province of British Columbia alone, these invasive hawkweeds are estimated to have eventual economic impacts in the tens of millions of dollars if they continue to spread at current rates (Frid et al. 2009), and in the United States an estimated $58 million per year is lost in resource revenues and in hawkweed control spending (Wilson 2002).

**History of the hawkweed biological control programme**

Investigations into natural enemies of *Pilosella* hawkweeds by scientists at CABI Europe-Switzerland were originally conducted as part of the biological control programme for invasive hawkweeds in New Zealand (Cortat et al. 2012). The five insects released in New Zealand, *Macrolabis pilosellae* (Diptera: Cecidomyiidae), *Oxyptilus pilosellae* (Lepidoptera: Pterophoridae), *Cheilosia psilophthalma* and *C. urbana* (Dipetea: Syrphidae), and *Aulacidea subterminalis* (Hymenoptera: Cynipidae), were all screened as potential agents for invasive hawkweeds in North America. The species *M. pilosellae* and *O. pilosellae* were both determined not sufficiently host-specific for North American release, as they also attack members of the true *Hieracium*, which are present in North America but do not occur in New Zealand. Results concerning *Cheilosia psilophthalma*'s host-specificity have been inconclusive and further investigations have been postponed. However, a petition for release of *C. urbana*, which under physiological host range testing appears to have a preference for *P. aurantiaca* (Grosskopf-Lachat and Parepa 2009), is currently in preparation (Cortat et al. 2012). *Cheilosia urbana* has been shown to attack a number of native North America *Hieracium* species at low levels in multiple-choice open-field tests (i.e in the presence of the target weeds, Grosskopf et al. 2006, Grosskopf and Parepa 2009). Thus, the likelihood of approval of this agent for release into North America is not certain. Further investigations were conducted with another species of

*Aulacidea, A. hieracii,* but this species rarely attacked the target weeds (Cortat et al. 2012) so investigations have ceased.

The only currently approved biocontrol agent for invasive hawkweeds in North America is the gall wasp, *Aulacidea subterminalis* Niblett (Hymenoptera: Cynipidae), which was approved for release in early 2011. The ecological host range of *A. subterminalis* consists of only *Pilosella officinarum*, but has been shown to develop on *P. flagellaris, P. aurantiaca* and *P. floribunda* in lab and/or common garden trials (Grosskopf et al. 2008). Based on the ecological and physiological host range data, it is unclear if *A. subterminalis* will establish in the introduced range, especially in British Columbia, where its only ecological host, *P. officinarum* is targeted for (chemical) eradication. As this method seems to be achieving success, *P. officinarum* is not targeted for biological control in British Columbia. As *P. flagellaris* is widely believed to be a stabilized hybrid species of *P. officinarum* x *P. caespitosa* (Bräutigam and Greuter 2007), if *A. subterminalis* were to establish on any of the invasive *Pilosella*, this species would likely be the most susceptible to attack by *A. subterminalis.*

At present, no currently approved agent is available for the remaining *Pilosella* species, including *P. caespitosa, P. glomerata, P. praealta, P. piloselloides,* and depending on the establishment success of *A. subterminalis,* also for *P. aurantiaca* and *P. floribunda*. Widely-recognized as highly invasive in western North America and the original target of the hawkweed biocontrol program for North America, *P. caespitosa* remains without a biological control agent. The same is true for its stabilized hybrid offspring *P. glomerata* (*P. caespitosa* x *P. cymosa*; Bräutigam and Greuter 2007), which since its detection in 2001 (Wilson et al. 2006) has been found to be widely distributed in south-eastern British Columbia. Consequently, there is still great interest in finding a biological control agent for *P. caespitosa* and *P. glomerata*, among the other invasive species of *Pilosella* in North America.

### *Aulacidea pilosellae,* a candidate biological control agent for invasive *Pilosella*

The gall wasp *Aulacidea pilosellae* Kieffer (Hymenoptera: Cynipidae) is the only remaining candidate insect classical weed biological control agent for *Pilosella* hawkweeds of European origin invasive in North America. This cynipid is a small uni-to bi-(tri-)voltine (number of generations per year) gall inducer, known to develop on select members of the genus *Pilosella* (Dalla Torre and Kieffer 1910, Ionescu 1957, Eady and Quinlan 1963, Buhr 1964, Houard

1913). Most literature records are from western Europe and mention only *Hieracium pilosella* (syn. *Pilosella officinarum*), as a host plant for *A. pilosellae* (Dalla Torre and Kieffer 1910, Ionescu 1957, Eady and Quinlan 1963). Fewer sources cite other host plant species, but records for the *Hieracium* synonyms of other *Pilosella* species are known, including *H. cymosum*, (*P. cymosa*), *H. echoides* (*P. echioides*), *H. flagellare* (*P. flagellaris*) and *H. floribundum* (*P. floribunda*) (Buhr 1964) and *H. florentinum* (*P. piloselloides*) (Houard 1913). However, one must interpret the published host range of *A. pilosellae* with some caution. The majority of the sources listed above were authored by entomologists and zoologists rather than hawkweed specialists and considering the complexity of *Pilosella* identification, the accuracy of these records should be approached with caution. Further, a number of often contradictory taxonomic treatments for *Pilosella* hawkweeds that have varied over time and space have been used in Europe (Šingliarová et al. 2011), which may make some records difficult to interpret. What can be gleaned with relative certainty is that *P. officinaum* is a common host of *A. pilosellae*, due to the large number of literature records and that there are likely multiple host species, all within the genus *Pilosella*.

As a part of the hawkweed biocontrol program for New Zealand, searches for phytophages associated with *P. officinarum* were conducted in the German Upper Rhine Valley and Black Forests and the Swiss Jura (hereafter called the 'Southern Range'), in 1993 by CABI Europe-Switzerland (Grosskopf 1994). *Aulacidea pilosellae* was found during these initial investigations on the leaf mid-ribs of *P. officinarum*, and when reared through to adulthood, only female wasps emerged, and parthenogenesis was suspected. These *A. pilosellae* also were observed to be bi-voltine. Galls of *A. subterminalis* were found on the stolons of *P. officinarum*. Due to the biocontrol programme's preference for insects attacking stolons, work was continued with *A. subterminalis* but not with *A. pilosellae* (Grosskopf 1994, 1995, 1996).

As part of hawkweed biocontrol programme for North America, searches for phytophages associated with *P. caespitosa* were conducted in the Czech Republic, south-west Poland and eastern Germany (hereafter the 'Northern Range') by CABI Europe-Switzerland beginning in 2001 (Grosskopf et al. 2002). These collecting trips revealed *A. pilosellae* commonly galled *P. caespitosa* and *P. glomerata*, both previously unrecorded hosts, as well as occasionally galled *P. cymosa*, *P. piloselloides* subsp. *bauhini*, and *P. floribunda*. Of further interest is that during the collection years 2001-2003, when multiple species listed above as hosts co-occured at field sites,

galls of *A. pilosellae* were often found on only one of the co-occuring species, i.e. galls were found on *P. cymosa* and not *P. glomerata* at a site even though *P. glomerata* was encountered as a frequent host plant, and on *P. glomerata* at a site and not *P. caespitosa*, even though *P. caespitosa* is also a frequent host (Grosskopf et al. 2002, 2003, 2004). Wasps from these collections also were reared through to adulthood, and a mixture of male and females emerged. In addition, individuals collected as larvae within galls from the Northern Range required a full year to emerge while individuals collected in the Southern Range completed two generations per year, as has also been reported in France (Eady and Quinlan 1963).

Physiological host range testing was conducted separately for both populations (i.e., Southern vs Northern Ranges) of *A. pilosellae*, and revealed disjunct host-ranges (Grosskopf et al. 2002, 2003, 2004). *Aulacidea pilosellae* collected from *P. officinarum* in the 'Southern Range' was able to develop, under no-choice conditions, on the field host *P. officinarum*, and additionally on *P. aurantiaca* and *P. piloselloides*. In contrast, *A. pilosellae* collected from predominately *P. glomerata* and *P. caespitosa*, but also possibly originating from *P. cymosa* and *P. piloselloides* subsp. *bauhini*, were able to develop, again under no-choice conditions, on the field hosts *P. caespitosa*, *P. glomerata* and *P. piloselloides*, and on *P. aurantiaca* but did not accept *P. officinarum* as a host.

The combination of the field collections, no-choice host-range testing and biological evidence of differences in sex-ratios and voltinism provided substantial ecological evidence to hypothesize that two biotypes of this insect likely exist. Further, the patterns of host-plant attack in the Northern Range indicate *A. pilosellae* may demonstrate additional patterns of host preference among other species of *Pilosella* hawkweeds.

## RESEARCH AIMS

The aims of this thesis are to first assess if *Aulacidea pilosellae* exhibits definable patterns of host-preference among species of *Pilosella* within its ecological host range, by conducting systematic and wide-spread surveys across the known European distribution of *A. pilosellae*, and to further quantify the ecological host range of *A. pilosellae* across the native European range. Determining if any such patterns of ecological host preference exist among *A. pilosellae* in the

native range would inform the hawkweed biological control programme as to which species of *Pilosella* would likely be controlled by *A. pilosellae* if approved for release in North America. Second, molecular methods are employed, specifically DNA sequencing of multiple gene regions, to determine if there is genetic divergence between the two purported biotypes of *A. pilosellae*, and if such genetic divergence occurs, if it is substantial enough to warrant considering that *A. pilosellae* may represent a cryptic species complex. If multiple cryptic biotypes or species were discovered, these cryptic types/species would then continue to be tested separately. If results of host-specificity (and/or efficacy) differ, as in the preliminary physiological host-range testing, molecular genetic evidence of cryptic types/species would likely facilitate treatment of the types/species as separate candidate agents. Such agents may exhibit narrower host ranges and may thus be more likely approved for release. The *A. pilosellae* in this study also were tested for infection with the bacterial endosymbiont *Wolbachia*, which if present, may be related to the observed parthenogenesis in the Southern Range population or other biological attributes of this species that could be informative to the biological control programme.

**Table 1.1:** Basic and hybrid species of European *Pilosella* hawkweeds known as exotics in western North America, following Wilson et al. (2006) and Wilson (2006, revised 2007), species names adapted based on the classification of Bräutigam and Greuter (2007) and the corresponding *Hieracium* synonym obtained from the Euro+Med Plantbase Project (Bräutigam and Greuter, 2007-09). Wilson et al. (2006) refers to *H. bauhini* (syn. *H. praealtum*) as present whereas Wilson (2006, revised 2007) refers to *H. praealtum* (syn. *H. bauhini*) as present. Both of these taxa are now recognized as sub-species in the same species aggregation and no longer as synonyms. We thus accept Wilson (2006, revised 2007) and show only *P. piloselloides* subsp. *praealta*.

| *Pilosella* species | Homotypic synonym/Basionym | Species designation | Postulated parental species |
|---|---|---|---|
| *Pilosella aurantiaca* (L.) F. W. Schultz & Sch. Bip. | *Hieracium aurantiacum* L. | basic | |
| *Pilosella caespitosa* (Dumort.) P. D. Sell & C. West | *Hieracium caespitosum* Dumort. | basic | |
| *Pilosella flagellaris* (Willd.) Arv.-Touv. | *Hieracium flagellare* Willd. | hybrid | *Pilosella caespitosa<>officinarum* |
| *Pilosella floribunda* (Wimm. & Grab.) Fr. | *Hieracium floribundum* Wimm. & Grab. | hybrid | *Pilosella caespitosa<>lactucella* |
| *Pilosella glomerata* (Froel.) Fr. | *Hieracium glomeratum* Froel. | hybrid | *Pilosella caespitosa<>cymosa* |
| ǂ*Pilosella lactucella* (Wallr.) P. D. Sell & C. West | *Hieracium lactucella* Wallr. | basic | |
| *Pilosella officinarum* Vaill. | *Hieracium pilosella* L. | basic | |
| *Pilosella piloselloides* (Vill.) Soják | *Hieracium piloselloides* Vill. | basic | |
| *Pilosella piloselloides* subsp. *praealta* (Gochnat) F. W. Schultz & Sch. Bip. | *Hieracium praealta* Gochnat | basic subspecies | |

ǂ known only from a single location

# CHAPTER 2: QUANTIFYING PATTERNS OF HOST PLANT SPECIES USE BY *AULACIDEA PILOSELLAE*, A CANDIDATE WEED BIOCONTROL AGENT FOR *PILOSELLA* HAWKWEEDS, IN ITS NATIVE RANGE

## SYNOPSIS

The aim of this research chapter is to address the question: Does the gall wasp *Aulacidea pilosellae* demonstrate any observable patterns of host-preference among species of *Pilosella* hawkweeds within its ecological host range (EHR), and if so, are host use patterns consistent among populations of hawkweeds? The secondary objective is to provide a description of the known ecological host range of *A. pilosellae* in its native distribution of central Europe.

There are at least nine species of the genus *Pilosella* Vaill. (Asteraceae: Lactuceae) that have been introduced into western North America (Table 1.1) where they outcompete native and cultivated plant species. If invasive hawkweeds continue to spread at current rates and are not controlled, the cost to the Province of British Columbia in economic losses in the forestry, rangeland, agricultural, recreational and real estate sectors is estimated to be $12-20 million dollars annually by 2020 (Frid et al. 2009). The North American Hawkweed Biocontrol Consortium was formed in order to fund overseas exploration for and testing of candidate biological control agents for invasive *Pilosella* hawkweeds, and investigations began in 2000.

*Aulacidea pilosellae* is a candidate classical weed biological control agent for *Pilosella* hawkweeds of European origin that are invasive in North America. *Aulacidea pilosellae* induces small (2-4mm) uni-locular galls, often several galls in a row, most commonly on the abaxial rosette leaf midribs, and occasionally on the stems and the stolons of multiple *Pilosella* species. As is typical for Cynipids and other gall formers, larval feeding diverts nutrients away from plant growth and reproductive potential. The host range of *Aulacidea pilosellae*, as reported in the European literature in the first half of the twentieth century, includes *Pilosella officinarum* (syn. *Hieracium pilosella*; Dalla Torre and Kieffer 1910, Ionescu 1957, Eady and Quinlan 1963); *P. cymosa (H. cymosum*), *P. echoides* (*H. echioides*), *P. flagellaris* (*H. flagellare*), *P. floribunda* (*H. floribundum*) (Buhr 1964); and also possibly all or some members of the *P. piloselloides* aggregation, due to the mention of *H. florentinum* (syn. *P. piloselloides*), as a host by Houard (1913). However, due to the complex and often overlapping morphology of *Pilosella*

hawkweeds, the number of different taxonomic treatments used in Europe during this period, and the fact that most of these authors were entomologists and not botanists, the host range documented in this literature may not be accurate or complete.

Exploration surveys for candidate agents for hawkweeds invasive in New Zealand, conducted by CABI Europe-Switzerland in the 1990s, documented *A. pilosellae* galling *P. officinarum* in the German Upper Rhine Valley and Black Forest and the Swiss Jura (hereafter called the 'Southern Range') (Grosskopf 1994). Further surveys for insects attacking *P. caespitosa* were conducted in the Czech Republic, south-west Poland and eastern Germany (hereafter the 'Northern Range'), and revealed *A. pilosellae* commonly galled *P. caespitosa* and *P. glomerata*, and occasionally galled *P. cymosa*, *P. piloselloides* subsp. *bauhini* and *P. floribunda*, and at one site, one individual *P. aurantiaca* plant was found to have three galls (Grosskopf et al. 2002, 2003, 2004). Grosskopf's records show that when multiple species of *Pilosella* documented as hosts at other locations co-occurred, galls of *A. pilosellae* were often found on only a select species. *Pilosella officinarum* was not mentioned to occur in the Northern Range, or if it was present, at least it was not surveyed by Grosskopf et al. during the early 2000s.

Because having an accurate and complete assessment of the ecological host range of *Aulacidea pilosellae* would be valuable to the biocontrol programme, we thus set out to systematically sample patterns of ecological host-use of *A. pilosellae* in its native central Europe. Our objective was to determine whether *A. pilosellae* demonstrates any preference for specific species of *Pilosella* within its host range and to further define its ecological host range. As the majority of *Pilosella* species invasive in western North America are abundant and common in central Europe, especially in the Czech Republic and surrounding areas, we focused our surveys in this region and also sampled populations in the German Black Forest and Swiss Jura.

We would identify hosts as "preferred" by *A. pilosellae* if those taxa were galled more than predicted by an appropriate null model. A proportional null model consists of *Pilosella* taxa being selected by *A. pilosellae* in proportion to their respective relative availability within a surveyed habitat (i.e., showing no preference). This lack of preference would express itself as patterns of host-use among *Pilosella* populations (field sites) that would only vary with the relative availability of hosts present at sites. We speculated that if patterns of host use by *A.*

*pilosellae* did significantly deviate from this null model (i.e. show a preference for particular host species of *Pilosella*), that *P. officinarum* would be favoured as it is frequently listed as a host in the mid-twentieth century European literature. More generally, on the basis of previous field research in the Northern Range (Grosskopf et al. 2002, 2003, 2004), we expected *P. caespitosa* and/or *P. glomerata* may also be favored as they were found to be common hosts of *A. pilosellae* in the Northern Range.

## METHODS

### Study area

The field research component of the study was conducted within the broad geographic distribution of *Aulacidea pilosellae* in central Europe (Figure 2.1). The study area was composed of four main sample regions: (S) the Jura Canton (*Canton du Jura*) of northwest Switzerland and the Black Forest (*Schwarzwald*) area in the *Baden-Württemberg* region of southwest Germany (Figure 2.2D), the area considered as the 'Southern Range';
(A) the Bavarian-Bohemian (*Bayerischer Wald/Zadní Bavorský les - Böhmerwald/Šumava*) forest region bordering eastern Germany and the southwestern Czech Republic, (B) the Ore Mountain range (*Erzgebirge*/*Krušné hory)*, bordering eastern Germany and the western Czech Republic, and (C) the Sudetes mountain range (*Sudeten*/*Sudety*) in the tri-border area of Germany, Poland and the Czech Republic, all considered as being in the 'Northern Range' (Figure 2.2).

### Sampling period

Field sampling was conducted during 5-30 June in regions A-C and 19-25 May, 28 June-2 July and 23-24 July 2010 in region S. As *A. pilosellae* has only one generation per year in the Northern Range, this June sampling period captured both the flowering period of the *Pilosella* hawkweeds (facilitating host identification) and allowed for gall collection. In the Southern Range, *A. pilosellae* has two generations per year, and thus sites were visited three times, with galls collected during the second two periods.

### Site selection and characteristics

Sites were selected using known sites identified in previous studies that confirmed the presence of target hawkweed species (G. Grosskopf-Lachat *pers. comm.*, F. Krahulec *pers. comm.*, Suda

et al. 2007) as well as sites located opportunistically. Our sites included localities where *A. pilosellae* had been both observed in the past and sites where its presence or absence was unknown. All field sites were semi-disturbed to heavily-disturbed herbaceous plant communities: the majority being managed semi-disturbed to disturbed open-pasture montane-meadows, characteristic of the regions surveyed, and the remaining sites being heavily disturbed roadside mixed-herbaceous plant communities, often near forest edges.

A total of 56 field sites were visited during the sampling season (Table 2.1). In the Northern Range, ten sites were visited in region A (A1-A10), eight in region B (B1-B8), and twenty-one in region C (C1-21), for a total of 39 sites. In the Southern Range, seventeen sites were visited in region S (S1-17), the first ten of which were located in the Black Forest and the latter seven in the Swiss Jura. Site S11 was eliminated from further consideration due to its proximity to CABI Europe-Switzerland which may have naturalized *A. pilosellae* from the Northern range in the common garden, in addition to the endemic *A. pilosellae* from the Southern range (G. Grosskopf-Lachat, *pers. comm.*), so data are reported for 16 of these sites, and the effective number of field sites was thus fifty-five. Site coordinates and elevation were estimated and recorded in the field using a Garmin 60CSx global positioning system. Site slope and aspect were measured and recorded using a clinometer and a digital compass, respectively. Exposure (in four classes from sheltered to open), and slope shape (concave, convex or straight) (Duncan et al. 1997) also were recorded. Site latitude and longitude, and associated abiotic data including elevation, slope, aspect, slope shape and canopy cover are summarized in Table 2.1.

**Estimation of *Pilosella* host plant availability**

Throughout our study, all *Pilosella* species we encountered were considered as possible hosts for *Aulacidea pilosellae* and surveyed without any bias as to those previously reported vs. unreported as hosts. However, post-survey, we defined ‘putative host *Pilosella* species’ as being those species utilized by *A. pilosellae* in this study (see Results and Discussion for further information) and created two categories of *Pilosella* species: all *Pilosella* species encountered at a site and only those considered as putative host *Pilosella* species.

In order to estimate *Pilosella* host plant availability, at each field site we used one of two survey methods, the choice of which depended upon the spatial distribution of hawkweed plants. Where density was low (less than 5-25% cover), a "transect method" was employed (see below). At

sites where the distribution of plants was more patchy (see below), a patch survey design was used which consisted of identifying hawkweed patches and sub-sampling multiple patches. The distribution was considered "patchy" if we observed at least 5-25% cover of at least one species of *Pilosella* over at least $5m^2$ in area, and subsequent patches of the same (or greater) density at least $0.1m^2$ in area per patch.

Transect method: The centroid of the site was determined by measuring the two axes of the site. Three 30m transects were then run from the centroid of the site radiating outwards towards the site edge. If any transect reached the site edge prior to reaching 30m in length, the remaining length was added to one of the remaining transects. The orientation of these transects was determined by selecting at random a heading between 1 and 360º. A single 0.1 $m^2$ (20x50 cm) Daubenmire quadrat (Daubenmire 1959) was placed every three metres along transects.

Patch survey method: *Pilosella* plants were assessed by sub-sampling multiple patches, up to a maximum of 20 patches per site. Patches were sub-sampled using the 0.1 $m^2$ (20x50 cm) Daubenmire quadrat (Daubenmire 1959). In order to determine the number and placement of quadrats within patches, patch size was calculated by measuring the long and short axis of the patch and estimating the area of the patch in square metres. In general, one quadrat was used as a sub-sample for a square metre of patch area, up to a maximum of 20 quadrats per patch. Quadrats were placed at randomly generated x,y locations in the patch.

Within quadrats (both survey methods), *Pilosella* plants were identified to species in the field using Sell and West (1976) and species descriptions compiled by G. Grosskopf-Lachat from S. Bräutigam and from the Hieracium Study Group at the Czech Institute of Botany (CIB). Any plants unidentifiable in the field were assigned a morph-species identification, pressed and collected. Both unidentified hawkweed plants and representative samples of field identified plants were collected and their identification verified by hawkweed experts F. Krahulec and J. Chrtek Jr. at the CIB. Voucher specimens of these pressed hawkweed specimens are stored at CABI Europe-Switzerland in Delémont, Switzerland.

Total *Pilosella* and putative host *Pilosella* species richness (species counts) for each site were obtained from quadrat surveying and are reported in Table 2.1. The mean, standard deviation and

range of richness for both the total and putative host *Pilosella* species were calculated and are shown as mean,SD(range).

*Pilosella* species' relative abundance were estimated as the percent foliar cover for each hawkweed species present in a quadrat, following Daubenmire's (1959) six cover categories: 0-5, 5-25, 25-50, 50-75, 75-95, 95-100, expressed as a percent, which were later converted to the cover midpoint: 2.5, 15, 37.5, 62.5, 85, 97.5, again expressed as a percent.

**Estimation of *Pilosella* host plant use by *Aulacidea pilosellae***

In order to survey for galls of *Aulacidea pilosellae, Pilosella* leaves, flowering stems and stolons were examined for galls on each hawkweed species within a quadrat. Due to the clonal nature of *Pilosella* hawkweeds, no attempt was made to discriminate individual plants. Individual leaves and stems were assessed for galling by recording the total leaf and stem counts for each *Pilosella* species within a quadrat, as well as the number of galled leaves and stems per species. When hawkweed leaves were particularly dense, only a portion of the quadrat was assessed (i.e. 25%) and the count extrapolated for the entire quadrat. The method of counting leaves/stems allowed for an assessment of the density of galls per species at a given site. The number of leaves/stems per species per site was not intended as a measure of the relative abundance of *Pilosella* species, as there is high variability in leaf size both between and among *Pilosella* species.

Once quadrat surveys were complete, galled plants were excised from the soil along with ~2-3 square inches of surrounding soil, potted in temporary pots in the field vehicle, and watered as needed. All live galled hawkweed plants were transported to the Czech Institute of Botany where their identification was verified by experts, with the exception of plants from site B1, as these plants rotted prior to verification; however, some galls remained intact. Galled plants were then live transported to CABI Europe-Switzerland where they were planted in pots and kept in mesh Aerarium® (http://www.wins.ch/aerarium.html) 60x60x180cm field cages, allowing *A. pilosellae* larvae to mature, over-winter and be used in host-specificity and other trials in subsequent studies.

**Analysis of host preference**

If particular host taxa were being selected by *A. pilosellae* more frequently than expected by an appropriate null model, we would accept this as evidence of host preference. The proportional

null model would consist of *Pilosella* taxa being selected by *A. pilosellae* in proportion to their respective relative availability within the surveyed habitats (i.e., showing no preference). This lack of preference would result in patterns of host-use among *Pilosella* populations (field sites) that would only differ based on the relative availability of hosts present at sites.

In the host preference analysis, only sites in the Northern Range regions (A.B,C) were used as the Southern Range (S) sites were dominated by a single *Pilosella* species (see Results), and this species was the only species ever galled in the Southern Range. It was reasoned that including these Southern Range sites in the preference analysis likely would obscure any pattern of preference expressed by *A. pilosellae* populations in the Northern Range. A proportional null model (and all other data analyses) was conducted in R Version 2.12.2 (R Development Core Team 2011) to determine whether patterns of host use deviated from a null expectation (i.e., in direct proportion to their relative availability) more than would be expected by chance.

### Geographic distribution of *Pilosella* host species

The relative abundance of each *Pilosella* species was estimated for each site and is shown as one pie chart per site, on the maps in Figure 2.2. All maps were generated in ArcGIS 10.0 (ESRI 2011), all data were projected using the Europe Albers Equal Area Conic with European Datum 1950 (ED50), and the countries layer converted from World Geodetic System 1984 (WGS 1984) to ED50. All map scales are approximate as distance is slightly distorted due to the projection.

## RESULTS

### Distribution of *Pilosella* host species availability

A total of 16 basic, sub- and stabilized hybrid species of *Pilosella* were surveyed for the presence of *Aulacidea pilosellae* across the survey area (Table 2.2). Additionally, in-situ hybrids of the recorded species were occasionally encountered and also surveyed, and are mentioned where relevant. In the Northern Range, all 16 of the encountered species were present at one or more sites, whereas in the Southern Range, only four of the 16 species were present (Table 2.3). Within the Northern Range, the *Pilosella* species composition was variable among both regions and sites within regions. Region A was characterized by *P. aurantiaca* and *P. officinarum*, while *P. caespitosa* and *P. glomerata* were both often present. Region B was characterized by *P. caespitosa*, while *P. aurantiaca* and *P. floribunda* were also often present. Notably, *P.*

*officinarum* was present at only one site and *P. glomerata* was not present in this region. Region C was characterized by *P. glomerata* and *P. officinarum*, while many other species were also somewhat common. The Southern Range/Region S was heavily dominated by *P. officinarum* and was present at all sites, while other species were rarely present (Table 2.3).

**Patterns of *Pilosella* species use by *Aulacidea pilosellae***

Across the four regions surveyed, *Aulacidea pilosellae* was detected at a minimum of one location in each sampling region. Across the Northern Range, a total of 15/39 sites and 11/16 sites in the Southern Range (Table 2.3) had the gall former present. In the Northern Range, *Aulacidea pilosellae* was found to gall *P. glomerata* and *P. caespitosa* at multiple sites and *P. floribunda*, *P. piloselloides* and *P. officinarum* at one site each. Over the Southern Range, *A. pilosellae* was only found to gall *P. officinarum*. We thus defined the 'putative host *Pilosella* species' to include *P. glomerata*, *P. caespitosa*, *P. floribunda*, *P. piloselloides* and *P. officinarum.*

In Region A of the Northern Range, galls of *A. pilosellae* were found at only one of 10 sites visited, on *P. glomerata*. In Region B, galls of *A. pilosellae* were found more commonly, at three of 10 sites, on *P. piloselloides*, *P. caespitosa* and *P. floribunda* at one site each. In Region C, galls of *A. pilosellae* were commonly found, at more than half of the 21 sites, on *P. glomerata* at eight sites, on *P. caespitosa* at two sites and on *P. officinarum* at one site. In the Southern Range/Region S, only *P. officinarum* was found with galls of *A. pilosellae*, at more than half of sites.

For any site that showed some attack by *A. pilosellae*, only one species of *Pilosella* was found to be galled. Thus, only a single species is listed as being galled at a given site in Table 2.3. It should be noted that a possible exception to this is site C3, which is listed as a galled site for *P. caespitosa* X *P. glomerata*. This is due to the plants from site C3 being described as a hybrid swarm of *P. caespitosa* and *P. glomerata*. As with the other sites, all galled plants were live-transported for *Pilosella* species confirmation by experts at the Czech Institute of Botany (CIB). Some plants from site C3 were described as resembling one postulated parental species more than the other, and more frequently were described as being more like *P. glomerata* (F. Krahulec, *pers. comm.*). Neither of the parental species was found at the site, which appeared to be a remnant of a once larger population as the hybrid plants were bordering a ditch on the edge

of a recently mowed field. Since the hybrid plants present represented a continuous entity at this site, no attempt could be made to determine the relative abundance of either *P. glomerata* or *P. caespitosa* – the plants appeared at the site to be one continuous entity, with only subtle differences in morphology detectable upon further inspection at the CIB. Thus, this site was considered to have only one 'species' in the preference analysis, and in further analyses this species was considered to be *P. glomerata* as a greater number of plants were described as being more like *P. glomerata* than *P. caespitosa*. A second exception is site C4, where 40 of the 144 galls were found on what was described as a hybrid of *P. caespitosa*, likely with *P. piloselloides*, but more closely resembling *P. caespitosa* (F. Krahulec, *pers. comm.*), thus this was considered as *P. caespitosa*. The remaining 104 galls at this site were found on *P. caespitosa*, and *P. piloselloides* was not found at the site.

**Analysis of host preference**

The null model we envisioned using in the host-preference analysis was not deemed appropriate upon examination of the data. In order to warrant the use of such a model, multiple host species would need to be utilized at at least some sites, which was not the case. Tables 2.3 and 2.4 clearly show that only one species of *Pilosella* was utilized per site. A further complication was that each site had a different number of putative host *Pilosella* species present (Table 2.1, 2.4), making comparisons across sites difficult (i.e. if all putative host *Pilosella* species were listed for all sites, the dataset would be primarily composed of zero values). Table 2.4 shows that where multiple putative host *Pilosella* species were present (i.e. where *A. pilosellae* had a 'choice' of multiple hosts) that in all cases either the most abundant or second most abundant putative host *Pilosella* species present was the host species utilized by *A. pilosellae*.

Our alternate hypothesis for this analysis was that the most abundant putative host *Pilosella* species present would be galled with greater frequency than the second most abundant putative host *Pilosella* species present, thus a one-tailed (greater) Fisher's exact test was employed using only those sites where more than one putative host *Pilosella* species was present. As only the most or second most abundant putative host *Pilosella* species was ever galled, we did not include the possibility of the third most abundant species (where present) being galled in our results. Consequently, we performed a 2x2 Fisher's exact test. Fisher's exact test (Fisher 1922, 1954) is appropriate for categorical data (i.e. the most abundant species being galled vs. the second most abundant species being galled), is robust to small samples size, and calculates the deviation from

the null expectation precisely. Our one-tailed Fisher's exact test demonstrated that when there were multiple putative host *Pilosella* species present at a site, that the most abundant putative host *Pilosella* species was galled by *A. pilosellae* significantly more often than expected by chance ($P = 0.028$, odds ratio = 10.24).

## DISCUSSION

### Host preference of *Aulacidea pilosellae*

We have demonstrated clear differences in patterns of host use of *Aulacidea pilosellae* across different localities. Specifically, we found that *A. pilosellae* in the Southern Range only attacks *P. officinarum*, the dominant species of *Pilosella* in this region. While a few other species of *Pilosella* (Table 2.3) were found in this region, none of these are reported as host species. In contrast, we found *A. pilosellae* in the Northern Range to attack multiple species of *Pilosella*: *P. glomerata*, *P. caespitosa*, *P. floribunda*, *P. piloselloides* and *P. officinarum*. Our study did not provide any direct evidence that *A. pilosellae* exhibits any preference for species of *Pilosella* in its' ecological host range. Instead, we found *A. pilosellae* to only utilize a single *Pilosella* species at any given site across the study area, and wherever there were multiple putative host species present in the Northern Range, the most abundant host species was utilized significantly more frequently than expected by chance.

Possible explanations for this pattern of frequency dependent attack could include (among other phenomena): local adaptation or host tracking of *A. pilosellae* to the most common host (Kaltz and Shykoff 1998, Lively and Dybdahl 2000), 'predator' switching (Cornell 1976), or cryptic host associations (Burns et al. 2008, Smith et al. 2008, Li et al. 2010, Toševski et al. 2011).

Local adaptation of insects to the most locally abundant host has been described in a number of systems (reviewed in Kaltz and Shykoff 1998). Lively and Dybdah (2000) tested whether local adaptation of a parasite to a host (snail) was due to disproportionate use of the common host (genotype) and found that local adaptation resulted from parasite tracking of locally common (sympatric) hosts, and that when parasites were exposed to allopatric host populations the pattern of host tracking disappeared. If this type of host tracking is the host selection mechanism used by *A. pilosellae*, this could explain why at each site only the most abundant host is used. Depending on the amount of time required for *A. pilosellae* to effectively track the most

abundant host in a novel habitat, this could have important implications for the biological control programme. If *A. pilosellae* requires several generations to host track in a novel environment, host use would be expected to be dispersed over multiple *Pilosella* species. This occurred when the parasite in Lively and Dybdah's (2000) system was exposed to novel populations. In contrast, if *A. pilosellae* tracks host availability rapidly, exclusive use of the most common host on a site would be expected to quickly appear in novel habitats.

Predator switching (Cornell 1976; also known as prey switching following Murdoch 1969; or apostatic selection, Clarke 1962) is a similar phenomenon to host tracking. This hypothesis is centred on the notion that predators (or parasites) follow two types of search strategies; undirected or directed. Undirected searching is used by generalist predators who accept prey items in proportion to their relative abundance (similar to our original null hypothesis of host use by *A. pilosellae*). Directed searching is done by obligate specialists and switching generalists, who benefit from search behaviour that increases the predator's ability to select the prey that promotes the highest fitness. Directed searching can result from maximizing either or both prey item value and prey abundance. The mechanism for this is referred to 'search image formation', whereby the predator follows a sensory cue given by the prey that maximizes predator efficiency, and is thought to become 'genetically fixed' over ecological time (Cornell 1976). Search image formation is also thought to be most common when prey items are patchy in distribution and predators are mobile (Cornell 1976), conditions met by the *A. pilosellae-Pilosella* system. If predator switching were the mechanism governing host selection by *A. pilosellae*, this would suggest that *A. pilosellae* is a 'switching generalist' among species of *Pilosella*. The implications of this type of host use pattern would be that *A. pilosellae* would likely adapt to novel distributions of hosts and continue to utilize the most abundant host in new habitats, unless the search image utilized has become genetically fixed among populations of *A. pilosellae*. This genetic fixation of the search image could be a possible mechanism behind cryptic host-associations, which are currently being revealed at a high rate in plant-insect systems (e.g. Burns et al. 2008, Smith et al. 2008, Li et al. 2010, Toševski et al. 2011). The potential for cryptic host-association of *A. pilosellae* is discussed in Chapter 3.

Of further interest are the contrasting patterns of usage of *P. officinarum* between *A. pilosellae* from the Southern Range and the Northern Range. *Pilosella officinarum* is the only suitable host that we encountered in the Southern Range and it was found to be galled by *A. pilosellae* at 69%

(11/16) of the sites where *P. officinarum* was present. In contrast, in the Northern Range we encountered *P. officinarum* at 16 sites yet it was found to be galled at only 6% (1) of those sites. Furthermore, at nearly half of these Northern Range sites where *P. officinarum* was present, *A. pilosellae* was also present, yet observed to gall other host species. While in 5 of these 7 cases the species galled was the most abundant, in the remaining two cases, *P. officinarum* was not the galled species despite being the most abundant potential host species present. Additionally, where we did find *P. officinarum* to be galled in the Northern Range (site C1), it was the only species of *Pilosella* present. These results of varying host use of *P. officinarum* by *A. pilosellae* cannot be easily explained by the patterns of frequency-dependent attack observed in all other populations/on other host species. If this pattern of frequency-dependent attack did hold true across all putative *Pilosella* host species, then *P. officinarum* should have been the utilized species at sites C4 and C6. One explanation for why *P. officinarum* was so rarely galled in the Northern Range could be that *A. pilosellae* has a weaker preference for *P. officinarum* than for other host species. Thus, if both *P. glomerata* and *P. caespitosa* are more preferred hosts than *P. officinarum*, this would explain why at sites C4 and C6 *P. caespitosa* and *P. glomerata* were respectively galled and not the more abundant *P. officinarum*. In order to test this hypothesis, either a greater number of sites where *A. pilosellae*, *P. officinarum* and either *P. glomerata* or *P. caespitosa* occurred would have to be sampled, or direct experimental evaluation of preference would be necessary. The exclusive use of *P. officinarum* by *A. pilosellae* in the Southern Range may simply be due to the absence of other more preferred species coupled with some selection on the gall wasp populations in this range to enhance either preference or performance.

**The ecological host range of *Aulacidea pilosellae***

Our results confirm that *P. officinarum* is a common host and thus far the only host of *A. pilosellae* in the Southern Range and a rare host of *A. pilosellae* in the Northern Range, despite *P. officinarum* being a common species in the Northern Range. Furthermore, we confirm that *P. glomerata* and *P. caespitosa* are common hosts of *A. pilosellae* in the Northern Range, and that *P. floribunda* and *P. piloselloides* are also acceptable but not common hosts. These results are consistent with those of Grosskopf, who similarly found *A. pilosellae* in the Northern Range to commonly gall both *P. glomerata* and *P. caespitosa*, and was the first to document these species as hosts of *A. pilosellae*. Her studies also commonly found *P. cymosa* to be a host species, whereas we did not locate this species on any of our study sites. Additionally, both *P. echoides* and *P. flagellaris* (*P. caespitosa*<>*P. officinarum*) have been reported as hosts of *A. pilosellae* in

previous literature (Buhr 1964). We did not encounter either of these species during our surveys, nor did Grosskopf report galling of these species, thus we cannot confirm if these species are within the ecological host range of *A. pilosellae*.

While our results confirm that *P. glomerata* and *P. caespitosa* are readily accepted hosts of *A. pilosellae* in the area we surveyed, we found *P. floribunda* and *P. piloselloides* to be utilized only once each. When we did find *P. floribunda* to be galled at one site, it was only one plant with three galls. Grosskopf also detected *A. pilosellae* on *P. floribund*a and it is mentioned as a host by Buhr (1964), so our record is not the first. We did encounter *P. floribunda* at eight sites in the Northern Range, and *A. pilosellae* was found at three of these sites, but only once on *P. floribunda*, when it was the most abundant species. Because of this we suspect that *P. floribunda* is only an occasional host for *A. pilosellae*, but suggest that if *A. pilosellae* does select hosts in a frequency-dependent manner, if *P. floribunda* were more locally abundant it could be a common host.

The *P. piloselloides* species aggregation is among the most difficult of *Pilosella* species complexes to resolve and has been subjected to many taxonomic revisions varying in the number of basic and sub-species described. *Pilosella piloselloides* is now treated as a basic species aggregation, including members recognized as distinct species in other treatments, including *P. piloselloides* subsp. *bauhini* and *P. piloselloides* subsp. *praealta* (Bräutigam and Greuter 2007). While we found *A. pilosellae* galls on a single plant clearly identified to be in the P. *piloselloides* aggregation (and based on our determination, assigned to the basic species *P. piloselloides*), this plant desiccated prior to having its identification confirmed, so we cannot definitively determine the species/sub-species. Grosskopf's records show collections from *P. piloselloides* subsp. *bauhini* (which at that time was being recognized as a separate species, *H. bauhini*) and also from plants determined to be either *P. piloselloides* or *P. piloselloides* subsp. *bauhini*. As well, Houard (1913) mentions *H. florentium* (syn. *P. piloselloides*) as a host of *A. pilosellae*. Thus, we are confident in our assessment that *A. pilosellae* does utilize at least some members of the *P. piloselloides* aggregation as hosts, but which members remains to be definitively established, as does the frequency of use of this species.

While one wild individual *P. aurantiaca* plant was found attacked by Grosskopf et al. (2004), we did not find *P. aurantiaca* to be attacked despite searching 16 sites of *P. aurantiaca* in the

Northern Range, only two of which where *A. pilosellae* was present, but both of which where *P. aurantiaca* was more abundant that *P. glomerata*, the galled species. *Pilosella aurantiaca* was not documented as a host in the remainder of Grosskopf's studies, nor was it found as a host plant in this study, nor is it mentioned in the literature as a host species. Under no-choice caged conditions, Grosskopf et al. (2002, 2003, 2004) did find *A. pilosellae* originating from both the Southern and Northern Ranges to accept *P. aurantiaca* as a host, and successful gall formation to ensue, thus *P. aurantiaca* is clearly within the physiological host range of *A. pilosellae*. Interestingly, *Aulacidea subterminalis*, widely thought to be the sister species of *A. pilosellae*, has only one known ecological host, *P. officinarum*, yet under cage and greenhouse conditions has also successfully developed on *P. aurantiaca*. Whether either *A. pilosellae* or *A. subterminalis* are able to develop on *P. aurantiaca* in a natural setting, either in the native or introduced ranges, remains unclear. However, the absence of gall induction under natural conditions suggests that *P. aurantiaca* it is not a readily acceptable host for *A. pilosellae* and that *P. aurantiaca* is outside the 'normal' ecological host range of *A. pilosellae*, despite being within its physiological host range.

**Summary and further directions**

Our study is one of the first to attempt to systematically assess both patterns of host species use and the ecological host range of a candidate weed biological control agent in its native range, prior to introduction. This approach has yielded a number of findings relevant to increasing the understanding of the *A. pilosellae – Pilosella* system.

Due to previous observations that *A. pilosellae* seemed to use only one host species per site when multiple putative host species were present, we hypothesized that *A. pilosellae* may have distinct preferences for certain species of *Pilosella* within its ecological host range. Our data provide no evidence to support this hypothesis. Instead, we found what appears to be strong frequency dependent host use in the majority of populations. We found that wherever *A. pilosellae* had a 'choice' between multiple putative host species, that only the most abundant host was galled. The exception to this pattern was two cases when *A. pilosellae* was present at sites where *P. officinarum* was the most abundant putative host, yet the second most abundant putative host species was galled. Further, we found that *A. pilosellae* in the Southern Range utilized only *P. officinarum* whereas *A. pilosellae* in the Northern Range utilized multiple host species (though only one per site) even though *P. officinarum* was commonly available. Thus, our hypothesis

that *P. officinarum* may be a preferred host was also rejected. While we found *A. pilosellae* to gall *P. officinarum* at more than half the sites we visited in the Southern Range, we found the wasp to only gall *P. officinarum* at 6% of sites in the Northern Range, despite being present at two additional sites in sympatry with *A. pilosellae*. Based on the data presented in this chapter, reasons for the variation in use of *P. officinarum* between the Southern and Northern Ranges remains unclear, although we further explore this issue in Chapter 3.

Further investigations into the mechanism(s) influencing patterns of host use by *A. pilosellae* could be very informative for understanding the biology of the species and to the *Pilosella* biological control programme. In order to investigate if the patterns of frequency-dependent host selection we observed in the Northern Range hold, we would suggest fully-factorial experiments in mini-communities of multiple putative host *Pilosella* species, and that these experiments be run for several generations in an effort to detect the mechanism driving such patterns. While we realize this is beyond the scope and resources of the biological control programme, we suspect that a better understanding of patterns of host-selection of this candidate agent could be very useful in prioritizing control efforts if *A. pilosellae* were released in North America. We further suggest that molecular investigations be conducted to determine if there are any geographic or host-associated patterns of cryptic genetic diversity present among populations of *A. pilosellae*, and we begin to address this in Chapter 3. Our findings regarding the ecological host range and host use patterns have important implications for the biological control programme for invasive *Pilosella* hawkweeds and are discussed further in Chapter 4.

**Table 2.1:** Site location, abiotic data and species richness and density for all *Pilosella* species present at a site and only those species considered putative hosts, for all 56 sites sampled in the survey area. All abiotic data were measured in the centre of each site. Site ID letter refers to region: (A) Bavarian-Bohemian forest region (E Germany/SW Czech Republic), (B) the Ore Mountain range (E Germany/W Czech Republic), (C) the Sudetes mountain range (Germany/Poland/Czech Republic, and (S) the Jura Canton/the Black Forest (NW Switzerland/SW Germany). Latitude and longitude expressed in decimal degrees (DD.DDDDD), elevation (metres above sea level), aspect and slope (degrees), slope shape, canopy cover (0-25,25-50,50-75,75-100%). Species richness (sp. rich.) expressed as raw richness, species density (sp. den.) expressed as species richness/survey area).

| | | | | | | | | | All *Pilosella* | | Putative host *Pilosella* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site ID | latitude | longitude | elev-ation | asp-ect | slo-pe | slope shape | can-opy cover | survey area | sp. rich | sp. den. | sp. rich | sp. den. |
| A1 | 49.00539 | 13.24235 | 591 | 193 | 5 | straight | 0-25 | 11.7 | 1 | 0.085 | 1 | 0.085 |
| A2 | 48.98666 | 13.18245 | 625 | 170 | 35 | straight | 0-25 | 317.7 | 2 | 0.006 | 1 | 0.003 |
| A3 | 49.19144 | 12.8572 | 450 | 259 | 15 | concave | 0-25 | 22.5 | 1 | 0.044 | 0 | 0.000 |
| A4 | 49.20778 | 12.87823 | 427 | 187 | 4 | convex | 0-25 | 5.6 | 1 | 0.178 | 0 | 0.000 |
| A5 | 48.92171 | 13.31238 | 818 | 161 | 30 | straight | 0-25 | 31.8 | 3 | 0.094 | 2 | 0.063 |
| A6 | 49.16453 | 13.27906 | 959 | 168 | 26 | concave | 0-25 | 33.6 | 1 | 0.030 | 0 | 0.000 |
| A7 | 49.15288 | 13.35002 | 877 | NA | 0 | straight | 0-25 | 86.9 | 4 | 0.046 | 2 | 0.023 |
| A8 | 49.18472 | 13.54583 | 866 | 282 | 10 | straight | 0-25 | 10.7 | 1 | 0.094 | 1 | 0.094 |
| A9 | 49.09754 | 13.43549 | 904 | 291 | - | convex | 0-25 | 14.0 | 2 | 0.143 | 1 | 0.071 |
| A10 | 49.08629 | 13.55598 | 1157 | 233 | 15 | convex | 0-25 | 122.8 | 4 | 0.033 | 2 | 0.016 |
| B1 | 50.32325 | 12.94927 | 457 | 180 | 30 | straight | 0-25 | 148.5 | 3 | 0.020 | 3 | 0.020 |
| B2 | 50.35435 | 12.93224 | 668 | 72 | 25 | convex | 0-25 | 125.0 | 2 | 0.016 | 1 | 0.008 |
| B3 | 50.41339 | 12.9228 | 1043 | 192 | 10 | straight | 0-25 | 2826 | 2 | 0.001 | 1 | 0.000 |
| B4 | 50.41768 | 12.9609 | 983 | 270 | 40 | convex | 0-25 | 240.0 | 2 | 0.008 | 1 | 0.004 |
| B5 | 50.4502 | 12.87026 | 770 | 214 | 40 | straight | 0-25 | 11.6 | 1 | 0.086 | 1 | 0.086 |
| B6 | 50.53475 | 12.85612 | 489 | 21 | 45 | convex | 0-25 | 164.2 | 2 | 0.012 | 1 | 0.006 |
| B7 | 50.55867 | 12.96361 | 592 | 176 | 25 | convex | 0-25 | 18.9 | 1 | 0.053 | 1 | 0.053 |
| B8 | 50.57201 | 13.23319 | 748 | 83 | 10 | convex | 0-25 | 161.9 | 3 | 0.019 | 3 | 0.019 |
| C1 | 51.05484 | 14.95759 | 192 | 190 | 15 | straight | 0-25 | 1585 | 1 | 0.001 | 1 | 0.001 |
| C2 | 50.82936 | 15.69646 | 367 | 130 | 5 | convex | 0-25 | 38.4 | 4 | 0.104 | 4 | 0.104 |
| C3 | 50.8251 | 15.72523 | 371 | 212 | 25 | convex | 0-25 | 96.8 | 2 | 0.021 | 2 | 0.021 |
| C4 | 50.80211 | 15.77237 | 460 | 228 | 5 | straight | 0-25 | 228.7 | 5 | 0.022 | 3 | 0.013 |
| C5 | 50.80051 | 15.82911 | 455 | 268 | 5 | straight | 0-25 | 34.1 | 1 | 0.029 | 0 | 0.000 |
| C6 | 50.69199 | 15.78967 | 930 | 125 | 15 | straight | 0-25 | 860.8 | 4 | 0.005 | 2 | 0.002 |
| C7 | 50.6898 | 15.76631 | 712 | 100 | 20 | straight | 0-25 | 54.1 | 3 | 0.055 | 2 | 0.037 |
| C8 | 50.7366 | 15.79944 | 947 | 358 | 25 | straight | 0-25 | 92.1 | 6 | 0.065 | 2 | 0.022 |
| C9 | 50.78263 | 16.08654 | 502 | 92 | 5 | convex | 0-25 | 4.9 | 2 | 0.410 | 1 | 0.205 |
| C10 | 50.74479 | 16.13907 | 496 | 84 | 40 | convex | 0-25 | 24.7 | 1 | 0.041 | 1 | 0.041 |
| C11 | 50.63969 | 16.21823 | 453 | 233 | - | straight | 0-25 | 2826 | 1 | 0.000 | 1 | 0.000 |
| C12 | 50.60844 | 16.51464 | 417 | 330 | 15 | convex | 0-25 | 24.0 | 3 | 0.125 | 3 | 0.125 |
| C13 | 50.62986 | 16.44457 | 502 | 168 | 45 | straight | 0-25 | 31.4 | 1 | 0.032 | 1 | 0.032 |
| C14 | 50.3376 | 15.89393 | 264 | NA | 0 | straight | 0-25 | 10.2 | 1 | 0.098 | 1 | 0.098 |

**Table 2.1:** cont.

| | | | | | | | | | All *Pilosella* | | Putative host *Pilosella* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Site ID | latitude | longitude | elev-ation | asp-ect | slo-pe | slope shape | can-opy cover | survey area | sp. rich | sp. den. | sp. rich | sp. den. |
| C15 | 50.54845 | 15.92645 | 470 | 95 | 10 | straight | 0-25 | 2826 | 2 | 0.001 | 1 | 0.000 |
| C16 | 50.67737 | 15.76638 | 975 | 178 | 10 | straight | 0-25 | 37.3 | 2 | 0.054 | 2 | 0.054 |
| C17 | 50.66272 | 15.61354 | 786 | 226 | 40 | straight | 0-25 | 112.7 | 3 | 0.027 | 2 | 0.018 |
| C18 | 50.64798 | 15.57045 | 739 | 210 | 8 | straight | 0-25 | 16.5 | 1 | 0.061 | 1 | 0.061 |
| C19 | 50.69016 | 15.5066 | 850 | 125 | 12 | convex | 0-25 | 64.1 | 1 | 0.016 | 1 | 0.016 |
| C20 | 50.75421 | 15.23061 | 667 | 218 | 5 | convex | 0-25 | 38.7 | 4 | 0.103 | 2 | 0.052 |
| C21 | 50.6995 | 15.09763 | 470 | 270 | 35 | straight | 0-25 | 110.7 | 1 | 0.009 | 0 | 0.000 |
| S1 | 47.77626 | 7.74086 | 832 | 159 | 26 | straight | 0-25 | 25.9 | 1 | 0.039 | 1 | 0.039 |
| S2 | 47.73661 | 7.80943 | 493 | 213 | 30 | convex | 0-25 | 630.0 | 1 | 0.002 | 1 | 0.002 |
| S3 | 47.79584 | 7.83639 | 824 | 215 | 17 | convex | 25-50 | 48.8 | 1 | 0.020 | 1 | 0.020 |
| S4 | 47.78709 | 7.97188 | 750 | 129 | 18 | straight | 0-25 | 231.8 | 1 | 0.004 | 1 | 0.004 |
| S5 | 47.76233 | 8.07261 | 946 | 80 | 15 | convex | 0-25 | 367.7 | 2 | 0.005 | 1 | 0.003 |
| S6 | 47.68997 | 7.9473 | 813 | 249 | 45 | straight | 0-25 | 19.0 | 1 | 0.053 | 1 | 0.053 |
| S7 | 47.80424 | 8.00807 | 950 | 124 | 27 | straight | 0-25 | 210.7 | 1 | 0.005 | 1 | 0.005 |
| S8 | 47.74232 | 8.00881 | 842 | 190 | 42 | convex | 0-25 | 618.0 | 1 | 0.002 | 1 | 0.002 |
| S9 | 47.7555 | 8.08876 | 914 | 217 | 30 | straight | 0-25 | 98.4 | 1 | 0.010 | 1 | 0.010 |
| S10 | 47.73071 | 8.00009 | 802 | 105 | - | convex | 0-25 | 182.6 | 1 | 0.005 | 1 | 0.005 |
| S12 | 47.36883 | 7.3078 | 520 | 163 | 13 | straight | 0-25 | 19.0 | 1 | 0.053 | 1 | 0.053 |
| S13 | 47.29182 | 7.22211 | 600 | 115 | 45 | straight | 0-25 | 13.8 | 1 | 0.073 | 1 | 0.073 |
| S14 | 47.28981 | 7.22146 | 617 | 36 | 35 | convex | 75-100 | 8.4 | 1 | 0.119 | 1 | 0.119 |
| S15 | 42.27899 | 7.22898 | 749 | 39 | 30 | convex | 0-25 | 81.6 | 1 | 0.012 | 1 | 0.012 |
| S16 | 47.33331 | 7.37273 | 426 | 103 | 25 | straight | 0-25 | 2.9 | 1 | 0.347 | 1 | 0.347 |
| S17 | 47.32344 | 7.37731 | 457 | 30 | 30 | straight | 0-25 | 108.8 | 3 | 0.028 | 1 | 0.009 |

**Table 2.2:** Basic and hybrid species of *Pilosella* present at the 56 native range field sites during the 2010 survey, based on the classification of Bräutigam and Greuter (2007) and the corresponding *Hieracium* synonym obtained from the Euro+Med Plantbase Project (Bräutigam and Greuter, 2007-09).

| *Pilosella* species | Homotypic synonym/Basionym | Species designation | Postulated parental species |
|---|---|---|---|
| *Pilosella aurantiaca* (L.) F. W. Schultz & Sch. Bip. | *Hieracium aurantiacum* L. | basic | - |
| *Pilosella caespitosa* (Dumort.) P. D. Sell & C. West | *Hieracium caespitosum* Dumort. | basic | - |
| *Pilosella floribunda* (Wimm. & Grab.) Fr. | *Hieracium floribundum* Wimm. & Grab. | hybrid | *Pilosella caespitosa<>lactucella* |
| *Pilosella fuscoatra* (Nägeli & Peter) Soják | *Hieracium fuscoatrum* Nägeli & Peter | hybrid | *Pilosella aurantiaca<>caespitosa* |
| *Pilosella glomerata* (Froel.) Fr. | *Hieracium glomeratum* Froel. | hybrid | *Pilosella caespitosa<>cymosa* |
| *Pilosella iserana* (R. Uechtr.) Soják | *Hieracium iseranum* (R. Uechtr.) Fiek* | hybrid | *Pilosella caespitosa<>lactucella<>officinarum* |
| *Pilosella lactucella* (Wallr.) P. D. Sell & C. West | *Hieracium lactucella* Wallr. | basic | - |
| *Pilosella melinomelas* (Peter) Holub | *Hieracium melinomelas* Peter | hybrid | *Pilosella caespitosa<>officinarum<>piloselloides* |
| *Pilosella officinarum* Vaill. | *Hieracium pilosella* L. | basic | - |
| *Pilosella piloselliflora* (Nägeli & Peter) Soják | *Hieracium piloselliflorum* Nägeli & Peter | hybrid | *Pilosella caespitosa<>lactucella<>officinarum* |
| *Pilosella piloselloides* (Vill.) Soják | *Hieracium piloselloides* Vill. | basic | - |
| *Pilosella piloselloides* subsp. *bauhini* (Schult.) S. Bräut. & Greuter | *Hieracium bauhini* Schult. | basic subspecies | - |
| *Pilosella polymastix* (Peter) Holub | *Hieracium polymastix* Peter | hybrid | *Pilosella caespitosa<>piloselloides* |
| *Pilosella rubra* (Peter) Soják | *Hieracium rubrum* Peter | hybrid | *Pilosella aurantiaca<>officinarum* |
| *Pilosella scandinavica* (Dahlst.) Schljakov | *Hieracium scandinavicum* Dahlst. | hybrid | *Pilosella caespitosa<>cymosa<>lactucella* |
| *Pilosella stoloniflora* (Waldst. & Kit.) F. W. Schultz & Sch. Bip. | *Hieracium stoloniflorum* Waldst. & Kit. | hybrid | *Pilosella aurantiaca<>hoppeana* |

*basionym differs

**Table 2.3a:** Basic, sub- and stabilized hybrid species of *Pilosella* present at each site in the Northern Range. For each site, species present are denoted with an X, and those also galled by *Aulacidea pilosellae* are denoted by an O. Bolding denotes being the most abundant *Pilosella* species present. *Pilosella* species names are abbreviated: *P. aurantiaca = aur, P. caespitosa = cae, P. caespitosa* X *P. glomerata = caexglo P. floribunda = flo, P. fuscoatra = fus, P. glomerata = glo, P. iserana = ise, P. lactucealla = lac, P. melinomelas = mel, P. officinarum = off, P. piloselliflora = pilf, P. piloselloides = P. pilo, P. piloselloides* subsp. *baunini = bau, P. polymastix = pol, P. rubra = rub, P. scandinavica = sca, P. stoloniflora = sto*.

| | Region A | | | | | | | | | | Region B | | | | | | | | | | | | | | | | Region C | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | C21 |
| *aur* | | X | **X** | **X** | **X** | **X** | **X** | | | X | | X | X | X | | **X** | | | | | | | **X** | | | X | | | | | | X | | | | | **X** | **X** |
| *cae* | | | | | | | X | | | X | X | | | **X** | **X** | X | **O** | X | | **X** | | **O** | | | O | | | | | | | | | | | | | |
| *cae xglo* | | | | | | | | | | | | | | | | | | | | | **O** | | | | | | | | | | | | | | | | | |
| *flo* | | | | | | | | | | | | **X** | **X** | | | | | **O** | | X | | X | | | | | | | | X | | | | | X | | | **X** | |
| *fus* | | | | | | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *glo* | | | | | O | | | | X | | | | | | | | | | | X | | | | O | | O | **O** | **X** | | **X** | **X** | | **X** | **O** | **O** | **O** | **O** | X | |
| *ise* | | | | | | | | | | | | | | | | | | | | | | | | **X** | **X** | X | X | | | | | | | | | | | | |
| *lac* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | X | |
| *mel* | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | |
| *off* | **X** | **X** | | | X | | X | **X** | | **X** | | | | | | | | X | **O** | X | | X | | X | X | X | | | | X | | **X** | | X | | | | | |
| *pilf* | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | X | | | | |
| *pilo* | | | | | | | | | | | **O** | | | | | | | | | | | | | | | | | | **X** | | | | | | | | | |
| *bau* | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *pol* | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | |
| *rub* | | | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | | | |
| *sca* | | | | | | | | | **X** | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *sto* | | | | | | | | | | | | | | | | | | | | | | | | | | **X** | | | | | | | | | | | | | |

**Table 2.3b:** Basic, sub- and stabilized hybrid species of *Pilosella* present at each site in the Southern Range. For each site, species present are denoted with an X, and those also galled by *Aulacidea pilosellae* are denoted by an O. Bolding denotes being the most abundant *Pilosella* species present. *Pilosella* species names are abbreviated: *P. aurantiaca = aur, P. lactucealla = lac, P. officinarum = off, P. rubra = rub*.

| | Region S | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 | S10 | S12 | S13 | S14 | S15 | S16 | S17 |
| *aur* | | | | | | | | | | | | | | | | X |
| *lac* | | | | | X | | | | | | | | | | | |
| *off* | **X** | **O** | **O** | **O** | **O** | **O** | **O** | **O** | **X** | **O** | **X** | **O** | **O** | **X** | **O** | **X** |
| *rub* | | | | | | | | | | | | | | | | X |

**Table 2.4:** Relative abundance of each putative host *Pilosella* species at each site where *Aulacidea pilosellae* was found; when multiple putative hosts were present only the most abundant was galled (except C4 and C6). Host species cover/area and gall density is shown where applicable.

| | Putative host *Pilosella* species relative abundance | | | | | | host species average % | host species total area | gall density (galls/host | gall density (galls/host |
|---|---|---|---|---|---|---|---|---|---|---|
| Site | *caespitosa* | *floribunda* | *glomerata* | *officinarum* | *piloselloides* | species galled | cover | covered | sp. leaf) | sp. % cover) |
| A5 | 0 | 0 | **75** | 25 | 0 | *glomerata* | 27.141 | 3.387 | 0.148 | 0.811 |
| B1 | 13 | 0 | 0 | 0 | **87** | *piloselloides* | 11.060 | 15.426 | 0.038 | 0.633 |
| B7 | **100** | 0 | 0 | 0 | 0 | *caespitosa* | 30.785 | 5.828 | 0.014 | 0.487 |
| B8 | 16 | **79** | 0 | 5 | 0 | *floribunda* | 15.966 | 23.855 | 0.002 | 0.188 |
| C1 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 49.373 | 782.571 | 0.005 | 0.749 |
| C3 | 0 | 0 | **100** | 0 | 0 | *caeXglo* | 24.694 | 23.900 | 0.063 | 6.722 |
| C4 | 48 | 2 | 0 | **51** | 0 | *caespitosa* | 31.945 | 86.697 | 0.040 | 4.508 |
| C6 | 0 | 0 | 30 | **70** | 0 | *glomerata* | 3.470 | 29.870 | 0.180 | 3.170 |
| C7 | **70** | 0 | 0 | 30 | 0 | *caespitosa* | 18.133 | 2.033 | 0.105 | 2.427 |
| C8 | 0 | 0 | **80** | 20 | 0 | *glomerata* | 8.138 | 6.369 | 0.032 | 3.318 |
| C9 | 0 | 0 | **100** | 0 | 0 | *glomerata* | 17.168 | 0.837 | 0.021 | 0.175 |
| C16 | 0 | 0 | **75** | 25 | 0 | *glomerata* | 9.384 | 3.504 | 0.178 | 2.877 |
| C17 | 0 | 6 | **94** | 0 | 0 | *glomerata* | 12.885 | 13.915 | 0.175 | 2.328 |
| C18 | 0 | 0 | **100** | 0 | 0 | *glomerata* | 15.480 | 2.552 | 0.116 | 1.809 |
| C19 | 0 | 0 | **100** | 0 | 0 | *glomerata* | 7.039 | 4.513 | 0.549 | 15.202 |
| S2 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 17.058 | 107.476 | 0.001 | 0.176 |
| S3 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 35.386 | 17.268 | 0.002 | 0.085 |
| S4 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 40.292 | 93.377 | 0.002 | 0.273 |
| S5 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 46.500 | 170.957 | 0.004 | 0.280 |
| S6 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 57.433 | 10.889 | 0.004 | 0.122 |
| S7 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 47.531 | 99.934 | 0.004 | 0.379 |
| S8 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 46.071 | 284.814 | 0.001 | 0.065 |
| S10 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 53.345 | 97.383 | 0.010 | 0.787 |
| S13 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 90.357 | 12.424 | 0.250 | 3.597 |
| S14 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 43.470 | 3.665 | 0.003 | 0.046 |
| S16 | 0 | 0 | 0 | **100** | 0 | *officinarum* | 69.000 | 1.987 | 0.006 | 0.130 |

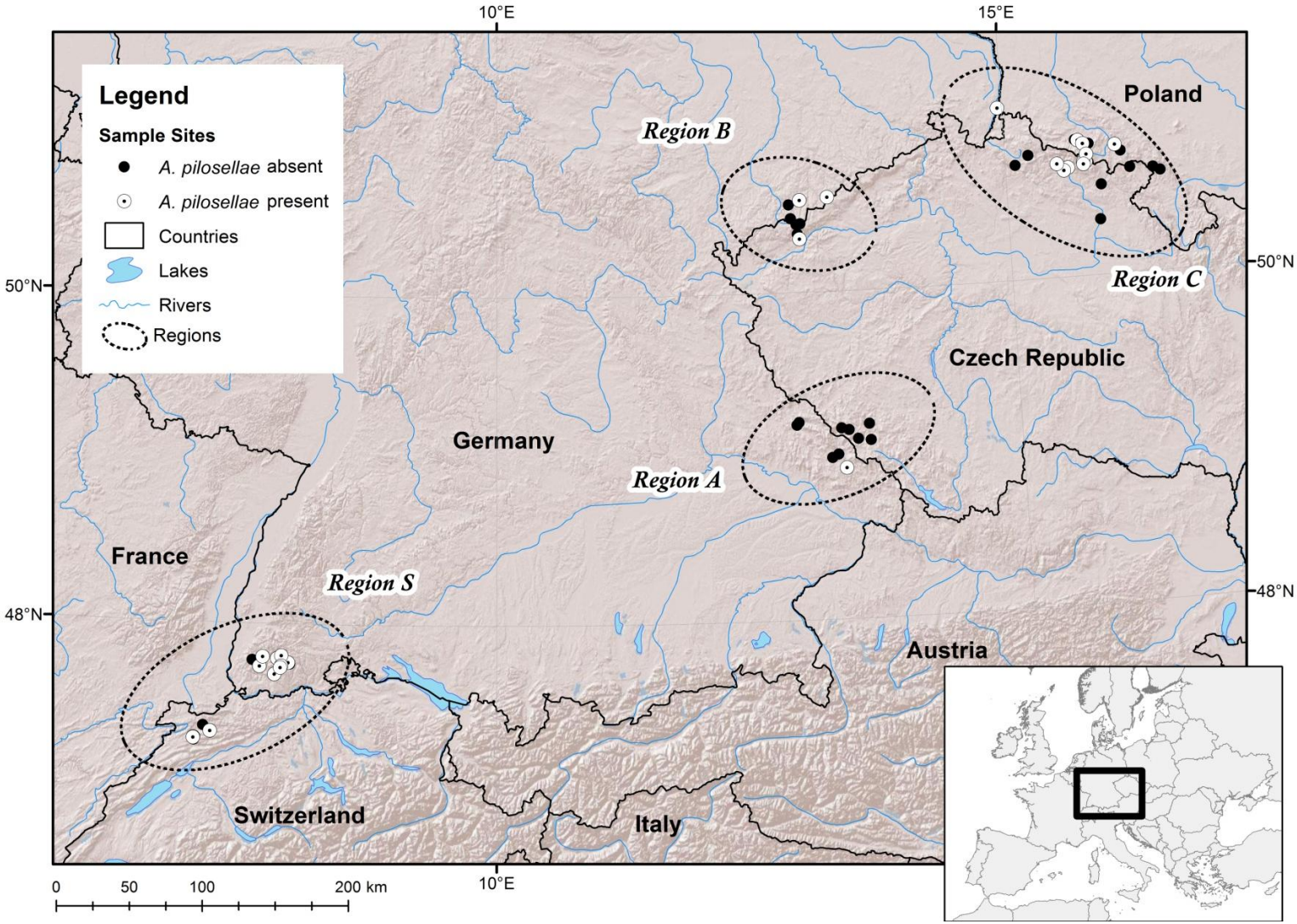


**Figure 2.1**: Study area comprising the central European distribution of *Aulacidea pilosellae*, divided into Regions A,B,C (Northern Range) and Region S (Southern Range), consisting of 55 sites of *Pilosella* spp., of which *A. pilosellae* was present at 26 and absent at 39. Map generated using ArcGIS 10.0 (ESRI 2011), with data projected using the Europe Albers Equal Area Conic with European Datum 1950; scale is approximate due to slight distance distortion.

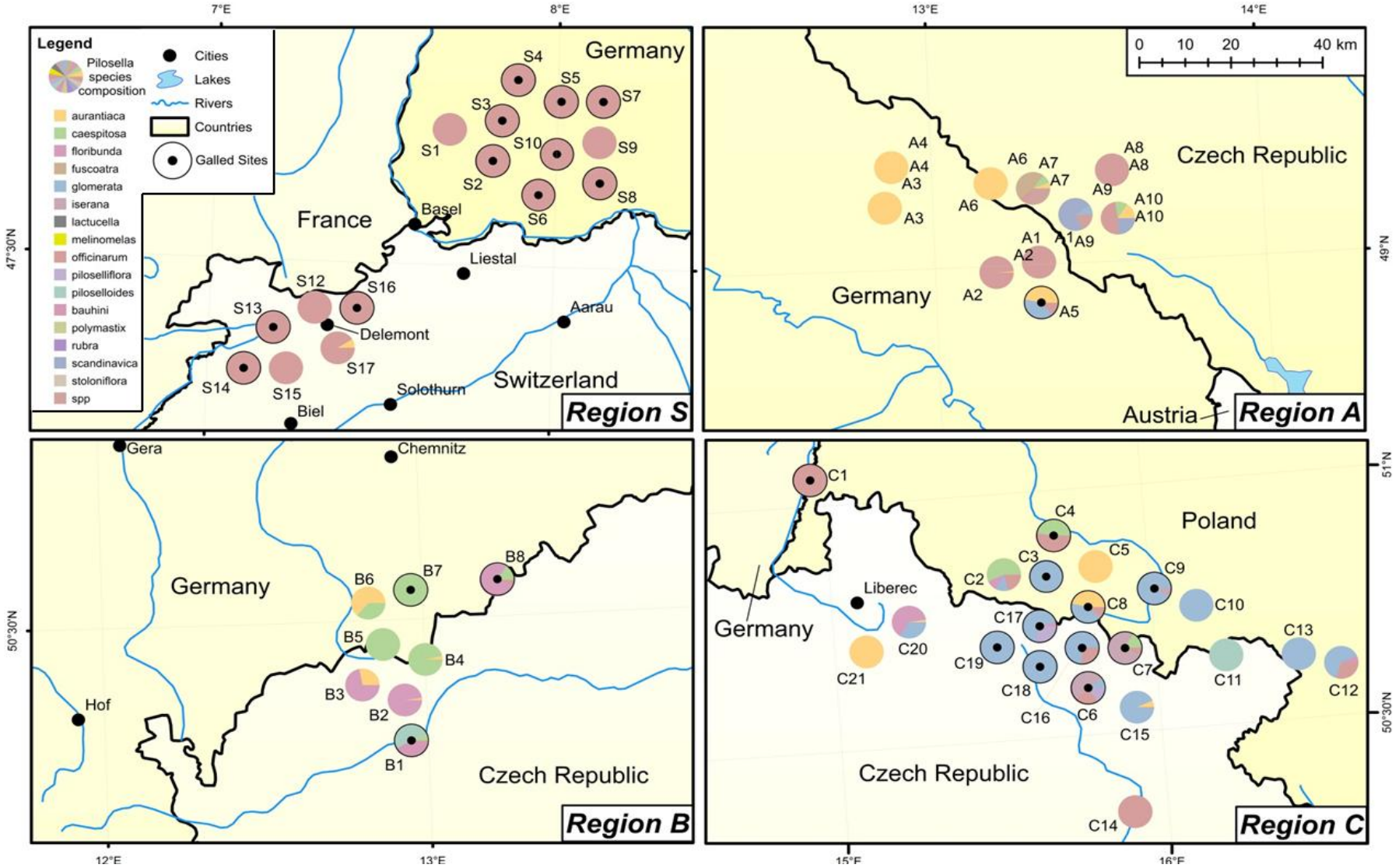


**Figure 2.2:** Each region of the study area (S, A, B, C) showing the species composition of each *Pilosella* sp. present at each site and the presence of *Aulacidea pilosellae* "Galled Sites". Map generated using ArcGIS 10.0 (ESRI 2011), with data projected using the Europe Albers Equal Area Conic with European Datum 1950; scale is consistent and approximate due to slight distance distortion.

# **CHAPTER 3:** CRYPTIC DIVERSITY OF A CANDIDATE WEED BIOLOGICAL CONTROL AGENT – DUE TO GEOGRAPHY, HOST-ASSOCIATION OR *WOLBACHIA* INFECTION?

## SYNOPSIS

The aim of this research chapter is to address the question: Is there genetic divergence between the two purported biotypes of the gall wasp *Aulacidea pilosellae*? If any patterns of genetic divergence within *A. pilosellae* are found, we further ask if these patterns are based on geographic distance (i.e. survey region sampled) or host plant use, and if there is sufficient variation to warrant considering that *A. pilosellae* may represent a cryptic species complex. We also test for the presence of the bacterial endosymbiont *Wolbachia*, as we hypothesize that *Wolbachia* infection, if present, may be related to the observed parthenogenesis in the Southern Range population.

*Aulacidea pilosellae* is a candidate classical weed biological control agent for *Pilosella* hawkweeds of European origin that are invasive in North America. *Aulacidea pilosellae* induces small (2-4mm) galls usually on the abaxial leaf midrib, and occasionally on the stems and stolons of multiple *Pilosella* species. Investigations conducted by Grosskopf et al. (2002, 2003, 2004) found *A. pilosellae* collected from the Northern Range (Figure 2.1) to exhibit a number of differences from *A. pilosellae* collected in the Southern Range (Figure 2.1). These differences included both the physiological host range, the number of generations per year, and the mode of reproduction.

Specifically, Grosskopf found *A. pilosellae* from the Northern Range to induce galls on *Pilosella glomerata*, *P. caespitosa*, *P. cymosa* and some members of the *P. piloselloides* complex, hereafter *A. pilosellae* ex. *Pilosella* spp. while *A. pilosellae* only galled *P. officinarum* in the Southern Range, hereafter *A. pilosellae* ex. *P. officinarum*. Further, she found *A. pilosellae* from the Northern Range has one generation per year and reproduces sexually, whereas *A. pilosellae* from the Southern Range has two generations per year and reproduces via parthenogenesis. When testing the physiological host range of the Northern Range populations, she found *A. pilosellae* ex. *Pilosella* spp. from the Northern Range will readily accept and heavily gall *P. glomerata* and *P. caespitosa*, will accept and gall (but less frequently and less heavily) *P.*

*aurantiaca* and *P. piloselloides* but will not accept *P. officinarum*. Conversely, when testing the Southern Range populations, she found that *P. officinarum, P. aurantiaca* and *P. piloselloides* were all accepted and galled with similar frequency, but that *P. caespitosa* and *P. glomerata* were not accepted.

Due to these combined differences in geographic location, voltinism, method of sexual reproduction and the physiological host range, she hypothesized that there were two biotypes of *A. pilosellae* and that differences in these biotypes were likely due to geographic separation (Grosskopf-Lachat, *pers. comm.*). Based on the host use patterns documented in Chapter 2 of this thesis, we also hypothesized that any patterns of cryptic genetic diversity may be associated with the host-plant used.

Having an accurate and complete assessment of any patterns of cryptic genetic differentiation of *Aulacidea pilosellae* would be valuable to the biocontrol programme. We thus set out to investigate the potential for cryptic genetic variation using DNA sequencing of *A. pilosellae* collected from multiple host plant species across the geographic distribution of *A. pilosellae* in its native central Europe. Our objective was first to determine whether *A. pilosellae* showed any patterns of cryptic genetic differentiation, second to determine if any observed patterns were based on geographic distance or host-plant species, and third to investigate if any members of the species we collected tested positive for infection with the bacterial endosymbiont, *Wolbachia*, which is known to induce parthenogenesis in hymenopteran hosts (Werren et al. 2008).

We sequenced three molecular markers, the cytochrome oxidase subunit 1 (CO1) gene region of mitochondrial DNA (mtDNA), the D2 region of the nuclear 28S ribosomal DNA (rDNA) gene region, and the internal transcribed spacer two (ITS2) gene region of the nuclear rDNA. We chose to sequence three markers for several reasons. First, while the CO1 and 28S regions are two of the most commonly used molecular markers to investigate sequence divergence in insects (Gariepy et al. 2007), there has been a strong acceptance in the literature that estimates of sequence divergence from multiple markers better estimate evolutionary relationships between taxa than phylogenies constricted from single markers (e.g. Caterino et al. 2000, Rokas et al. 2002b, Smith and Fisher 2009) due to differences in mutation rates/nucleotide substitution in different gene regions (Avise 1994). CO1 and 28S sequences were available for other members

of the genus *Aulacidea*, allowing us to compare divergence among other members of this genus. We also sequenced the ITS2 region because it is known to exhibit a degree of variation that is intermediate between the 28S-D2 region and the CO1 region in the Cynipids.

## METHODS

### Specimen collection

*Aulacidea pilosellae* were collected as larvae in galls on multiple *Pilosella* spp. from eight representative populations (Table 3.1) across the central European distribution of the insect. Each field site, which contained one to four species of *Pilosella* hawkweeds, was systematically searched following the methodology outlined in Chapter 2. Among the species of *Pilosella* examined (Table 2.2) galls of *A. pilosellae* were found on the host plants *P. glomerata*, *P. caespitosa*, *P. piloselloides*, *P. floribunda* and *P. officinarum* in the Northern Range and only on *P. officinarum* in the Southern Range. At least two specimens per host species (site) per geographic region (A, B, C, S) were sequenced at CO1 and 28S-D2 where sample size permitted (Table 3.1), however only one specimen per host species (site) per geographic region was sequenced at ITS2.

All galled plants collected in the Southern Range were determined to be *Pilosella officinarum*. Because whole plants of this species do not often survive transplanting (Grosskopf-Lachat, *pers. comm.*), galled leaves of *P. officinarum* were harvested and kept in a moistened environment until insect emergence a few weeks later. *Pilosella* plants from the Northern Range were assigned preliminary identification to species level in the field, and all galled plants were live-collected and transplanted to the Institute of Botany at the Academy of Sciences of the Czech Republic, where they were definitively identified by F. Krahulec and J. Chrtek Jr.. The only exception to this was for galled plants collected from site B1. These had decayed to the point where identification confirmation was not possible. However, galls remained intact and live larval *A.pilosellae* were dissected and preserved for DNA extraction. Transplanted plants were then transferred to sealed, fine mesh Aerarium® field cages measuring 60x60x180 cm (available from http://www.wins.ch/aerarium.html) at CABI Europe-Switzerland, where larvae developed within galls until the end of the plants' growing season. As larvae within galls do not always survive overwintering, especially after transplanting, a sub-sample of galls was dissected and 3$^{rd}$ instar larvae preserved in 95% EtOH to be used for DNA extraction. Wherever larvae

successfully overwintered and emerged as adults, reference specimens were reserved and are stored at CABI Europe-Switzerland.

Two additional larval specimens, collected by and determined as *A. pilosellae* ex *P. officinarum* by J.L. Nieves-Aldrey that were collected in 2006 from the Madrid Province, Spain were also used in the study. As well, two individuals of *Aulacidea subterminalis,* which is thought to be a close relative or even a conspecific of *A. pilosellae* (Nieves-Aldrey 1994, 2001) were obtained from a rearing colony (the original population was obtained in either the Swiss Jura or the German Black Forest) kept at CABI Europe-Switzerland, and were used as a species level out-group.

**DNA extraction, amplification and sequencing**

DNA was extracted using the QIAGEN DNeasy® Blood & Tissue Kit (Qiagen, Inc., Germany) according to the manufacturer's instructions, except elutions were done with 50uL (vs. the recommended 200uL) of elution buffer. In order to amplify the cytochrome oxidase subunit 1 (CO1) gene region of mitochondrial DNA (mtDNA), the forward primer lco1490 [5'- GGT CAA CAA ATC ATA AAG ATA TTG G-3'] and the reverse primer hco2198 [5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'] (Folmer et al. 1994) were used. To amplify the D2 region of the nuclear 28S ribosomal DNA (rDNA) gene region, the forward primer 28S-D2(F) [5'-CGT GTT GCT TGA TAG TGC AGC-3'] and the reverse primer 28S-D2(R) [5'-TCA AGA CGG GTC CTG AAA GT-3] (Heraty et al. 2004, Acs et al. 2010) were used. The internal transcribed spacer two (ITS2) gene region of the nuclear rDNA, was amplified using the forward primer ITS2-FWD [5'TGT GAA CTG CAG GAC ACA TG-3'] and the reverse primer ITS2-REV [5'-AAT GCT TAA ATT TAG GGG GTA-3'] (Campbell et al. 1993). The forward primer wsp 81F [5'-TGG TCC AAT AAG TGA TGA AGA AAC] and the reverse primer wsp 691R [5'-AAA AAT TAA ACG CTA CTC CA] (Zhou et al. 1998) were used to detect the presence of the endosymbiotic bacterium *Wolbachia*. All primers were diluted to 20uM.

PCR mastermix for all amplifications was made using TaKaRa LA Taq™ DNA Polymerase and its provided components. Each 25uL reaction consisted of: 2.5uL PCR buffer, 4.0 uL dNTPs, 0.5ul forward primer, 0.5uL reverse primer, 0.2uL LA taq, 16.3 uL PCR grade water, and 1uL template. PCR cycling conditions for CO1, 28S-D2 and ITS2 amplifications were performed following the step action times in Table 3.2, and for wsp following the detailed protocols for a

touchdown PCR profile as described in Kyei-Poku et al. (2005). All specimens tested for the presence of *Wolbachia* were run in a PCR alongside wsp amplified bacterial DNA obtained from the *Wolbachia* infected horn fly *Haematobia irritans*, a standard positive control for the presence of *Wolbachia* (Zhang et al. 2009). The PCR product from all reactions was visualized on a UV transilluminator and bands were excised quickly under low light conditions. Amplified product was gel purified using a QIAquick® Gel Extraction Kit (Qiagen, Inc., Germany) and quantified prior to sequencing. DNA with required primers was sent for sequencing to either the University of Calgary or McGill University.

**Sequence alignment and phylogenetic analyses**

All sequence data for the *A. pilosellae* and *A. subterminalis* specimens under study were edited and aligned using Geneious versions 5.3-5.6 (Drummond et al. 2011). Additional CO1 and 28S-D2 sequences for all publicly available sequenced members of the genus *Aulacidea* (*A. phlomica, A. hieracii, A. freesei, A. tragopogonis*) and a tribe-level outgroup, *Diplolepis rosae*, were obtained from GenBank (Benson et al. 2005), as was the ITS2 sequence for *Diplolepis rosae* (Table 3.3). ITS2 data were not available for other *Aulacidea* species. Sequence data from each gene region (CO1, 28S-D2 and ITS2) were aligned separately and neighbor-joining trees produced using the Tamura-Nei (TrN) method and 1000x bootstrapping in Geneious versions 5.3-5.6 (Drummond et al. 2011), using either *A. subterminalis* (ITS2) or *D. rosae* (CO1 and 28S-D2) as the specified out-group. The standard nucleotide Basic Local Alignment Search Tool (BLASTn) was used to search nucleotide databases (all GenBank and other databases; http://blast.ncbi.nlm.nih.gov) for the most similar available sequences to the three sequenced gene regions, using separate consensus sequences for each gene region, generated in Geneious. One additional CO1 sequence, obtained from GenBank, was added to the CO1 alignment based on the outcome of the BLASTn (Table 3.3).

Bayesian information criterion (BIC) model selection was performed independently for all gene regions in jModelTest (Posada 2008), which heavily uses Phyml (Guindon and Gascuel 2003). The sequences were then concatenated manually and a Bayesian phylogenetic inference was performed twice, once using a combined CO1-28S dataset and again using the combined CO1-28S-ITS2 three-gene region data set, in MrBayes v 3.2.0 (Ronquist et al. *in press*; Ronquist and Huelsenbeck 2003) and 50% majority-rule consensus phylogenies generated.

*Wolbachia* sequence data obtained were also edited and aligned using Geneious v 5.3, and BLASTn performed to find the most similar sequences. DNA sequences from this study will be submitted to the Barcode of Life Database (BOLD) and GenBank pending publication.

## RESULTS

### Sequence lengths and variability

The final edited alignment lengths for the CO1, 28SD2 and ITS2 gene regions for the *Aulacidea pilosellae* and *A. subterminalis* sequenced in this study were 643, 542 and 563 base pairs for each region respectively. Among the seventeen *A. pilosellae* sequences, the CO1 sequences showed a relatively high percentage of variable sites (44 of 643 or 6.8%) while the 28S sequences showed a very low percentage, with only one (0.002%) variable site, represented by a single transition in one individual sequence. The ITS2 region showed quite a low number of variable sites (8 of 563 or 0.014%), further discussed below.

### CO1 sequence divergence

The seventeen individuals sequenced at the CO1 gene region were obtained from nine collection sites (Table 3.1). Where more than one individual was sequenced per collection site, individuals from the same collection site shared an identical CO1 sequence, except for individuals collected from the site C16 (individuals L2a, L2b and L2c). L2b and L2c differ by a single base pair, whereas L2a differs from L2b and L2c by 17 base pairs (2.6%). The CO1 sequences delineate *A. pilosellae* into three distinct lineages (Figure 3.1), which will be referred to as *A. pilosellae* 1, *A. pilosellae* 2 and *A. pilosellae* 3, and indicate that the lineage *A. pilosellae* 1 is basal to the other two lineages. The lineage *A. pilosellae* 1 consists of individuals collected from both the Southern and Northern Ranges and has an intra-lineage sequence divergence of 0.55% (Table 3.4). Sequences which group together in this lineage are from the host plant species *Pilosella officinarum* (specimen L1 from site S3 in the Southern Range and specimens L4 (a,b), from site C1 in the Northern Range) and *P. floribunda* (specimen L5 from site B8 in the Northern Range). As well, the CO1 sequence obtained from the BLASTn, accession no. JN288739 (Table 3.3), also was placed in this lineage, with high branch support. As this individual was collected in a Malaise trap (A. Smith, *pers. comm.*), there is no host plant information available.

The second lineage *A. pilosellae* 2, has an intra-lineage sequence divergence of 2.5% (Table 3.4) and is composed of two individuals with identical CO1 sequences collected in Spain (ES), reportedly obtained from *P. officinarum* and one specimen (L2a), collected in the northern Czech Republic from *P. glomerata* at site C16.

The third lineage, *A. pilosellae* 3, has an intra-lineage sequence divergence of 1.1% (Table 3.4) and is composed of all of the individuals collected from the host species *P. caespitosa*, *P. piloselloides* and *P. glomerata*, except for the L2a individual (also collected from *P. glomerata*). Within this lineage, there are two branches. Individuals collected from *P glomerata* and *P. caespitosa* from sites C16 and C4 form one branch, whereas individuals collected from *P. glomerata*, *P. caespitosa* and *P. piloselloides* from sites B7, B1, and A5 form a second branch.

For the CO1 gene region sequenced, the average intra-lineage sequence divergence is 1%, while the average inter-lineage sequence divergence is 4%, with the greatest divergence being between the lineages *A. pilosellae* 1 and *A. pilosellae* 3. Figure 3.1 shows that at the CO1 gene region, *A. subterminalis* has the most similar sequence to *A. pilosellae* of any of the *Aulacidea* spp. for which CO1 data were available, with an average inter-specific sequence divergence of 9.4% between *A. pilosellae* and *A. subterminalis*, while there is 15.5% sequence divergence between *A. pilosellae* and *A. hieracii*. Figure 3.1 also shows that *A. tragopogonis*, *A. phlomica* and *A. freesei* have CO1 sequences that are quite similar, and that the CO1 sequence from *A. hieracii* is less similar.

**28S-D2 sequence divergence**

The lack of variation among the 28S gene region in the seventeen *A. pilosellae* individuals sequenced is shown in the neighbour-joining tree of the 28S-D2 sequences (Figure 3.2). This figure shows that *A. pilosellae* has a nearly uniform (except the individual with the single transition) 28S-D2 gene region that is distinct from all of the other species of *Aulacidea* for which a 28S gene region sequence is available for comparison. Among the sequences available in GenBank (*A. hieracii*, *A. tragopogonis*, *A. freesei* and *A. phlomica*), there is only one 28SD2 sequence available per species, so it is not possible to infer if it is the norm within this genus for a species to show a (nearly) uniform 28S-D2 sequence, although the two *A. subterminalis* specimens we sequenced also shared an identical 28S-D2 sequence. The single transition causes the average intra-lineage pairwise sequence divergence to be one tenth of a percent, due to the

divergence in the *A. pilosellae* 3 lineage (the single transition between the L2a and ES individuals), and causes the average inter-lineage variation to be 0.06% (note there is no divergence in the 28S sequence between lineages 1 and 3, only between lineage 2 when compared to either lineage 1 or 3). On average, there is 2.9% sequence divergence at 28S-D2 between all *A. pilosellae* individuals and *A. subterminalis* and nearly 4% divergence at 28S-D2 between *A. pilosellae* and *A. hieracii*.

**ITS2 sequence divergence**

The neighbour-joining tree for ITS2 (Figure 3.3) shows there is very little sequence divergence among the ten *A. pilosellae* specimens sequenced, with only an average inter-lineage sequence divergence of 0.44 % (Table 3.4). Four of the sequences (L1, ES, L2c and L7) are identical; three have single transitions at different positions (L3, L4, L6) and two (L2a and L2b) share a single transition, which is also shared with Asubt (*A. subterminalis*), and specimen L8 has three transitions at different positions. The ITS2 sequence we obtained from *A. subterminalis* differs substantially (14.9%, Table 3.4) from the sequences obtained from *A. pilosellae*. ITS2 sequences for any other *Aulacidea* species were not available for inclusion.

**Bayesian phylogenetic inference**

The Bayesian Inference Criterion (BIC) identified different best-fit models of nucleotide substitution for each gene region dataset. The GTR+G (general time reversible + gamma distribution; Lanave et al. 1984) model was identified as the model of best fit for the CO1 dataset, while the HKY (Hasegawa-Kishino-Yano; Hasegawa et al. 1987) and F81 (Felsenstein 1981; Felsenstein 1981) were identified for the 28S-D2 and ITS2 datasets, respectively. The MrBAYES 50% majority-rule consensus phylogeny combines data from multiple gene regions and conducts one phylogenetic analysis using the concatenated data and in this case, a combined GTR+G/HKY/F81 model for the CO1-28S-ITS2 analysis and a combined GTR+G/HKY model for the CO1-28S analysis. The intra- and inter-lineage sequence divergence from both of these concatenated multiple gene region datasets are shown in Figure 3.4, while only the MrBAYES 50% majority-rule consensus phylogeny (tree) for the three region dataset is shown (Figure 3.4), because it did not differ substantially from the tree generated based on the two gene region dataset.

The MrBAYES 50% majority-rule consensus phylogeny splits *A. pilosellae* into multiple lineages (Figure 3.4), as the CO1 data is incorporated as roughly one third of the combined data set. Thus, the arrangement of the *A. pilosellae* portion of the Bayesian phylogeny is similar to the CO1 neighbour-joining tree (Figure 3.1), although the arrangement of the branches/lineages differs. In the Bayesian phylogeny, the lineage *A. pilosellae* 2 (L2a and ES) is shown as basal to both *A. pilosellae* 1 and *A. pilosellae* 3. This combined phylogeny also places *A. subterminalis* and *A. hieracii* in a single lineage unlike the CO1 NJ phylogeny but more similar to the 28S-D2 phyhlogeny, and indicates that *A. pilosellae, A. subterminalis* and *A. hieracii* shared a common ancestor more recently than with any of the other *Aulacidea* species sequenced.

### *Wolbachia* infection

At least one individual of *A. pilosellae* per collection site was tested for the presence of the endosymbiont *Wolbachia*, as was one of the two *A. subterminalis* individuals sequenced. The *A. subterminalis* and two of the *A. pilosellae* individuals (L1 from site S3 in the Southern Range and L4 from site C1 in the Northern Range) tested positive for the presence of the endosymbiont *Wolbachia*. The sequence generated from the wsp amplified DNA was identical for all three specimens, and was compared to available sequences using BLASTn. Two existing *Wolbachia* sequences were highlighted as identical, both from the Pteromalidae, *Pteromalus purpanum* (accession no. DQ508543 and DQ493917) and *Nasonia vitripennis* (also accession no. DQ493917).

## DISCUSSION

The objective of this research was to test the hypothesis that there may be cryptic genetic divergence between the two purported biotypes of *Aulacidea pilosellae*. The initial hypothesis was that if any genetic divergence was found, it would be between *A. pilosellae* from the Southern Range and *A. pilosellae* from the Northern Range, due to observed differences in the physiological host range and life history between these biotypes. Our work rejects this hypothesis, not because genetic divergence was not found, but because it was not based primarily on geography. Perhaps our most important finding is that the *A. pilosellae* individuals collected from *P. officinarum* in the Southern Range share high sequence similarity with individuals collected from *P. officinarum* in the Northern Range. We do find evidence of host-associated divergence, our second hypothesis, and interpret that this host-associated divergence

splits *A. pilosellae* by host group – those that use *P. officinarum* (and to a lesser extent *P. floribunda*) are of one-two lineages, whereas those that use *P. glomerata*, *P. caespitosa* and *P. piloselloides* are of another lineage. Further, we detect *Wolbachia* only in those wasps collected from *P. officinarum*, thus we find our hypothesis of *Wolbachia* infection being related to the observed parthenogenesis in the Southern Range *A. pilosellae* to have some support.

**Differences in sequence variation in different gene regions**

Our results show that the sequence divergence varied substantially between each of the three gene regions of *Aulacidea pilosellae*. The most variable region was the CO1 region, which was the only region to divide *A. pilosellae* into multiple lineages. Although the NJ models for the 28S-D2 and ITS2 gene regions did not divide our *A. pilosellae* samples into lineages, when the same (CO1) lineage based comparisons were made for the 28S-D2 and ITS regions, the intra-lineage variation in the CO1 region (1.0%) was nearly twice that of the ITS2 region (0.58%) and two orders of magnitude higher than that of the 28S-D2 region (0.01). The average inter-lineage variation of CO1 (4.0%) was an order of magnitude higher than that of the ITS2 region (0.44%) and roughly two orders of magnitude higher than that of the 28S-D2 region (0.06%).

These results are in concordance with the findings of Rokas et al. (2002b), who tested eight loci (gene regions) to determine the most appropriate phylogenetic markers for Cynipid systematic studies. They demonstrated that the 28S region has a relatively low inter-specific percent sequence divergence (among species of all tribes of Cynipids) of 0.28 to 8.89% and is useful for identifying within family divergences (i.e. tribe level). They also observed that the ITS and CO1 regions are more useful for lower level phylogenetics (i.e. among and within genera), and have inter-specific percent sequence divergences of 1.33 to 16.69 and 6.68 to 20.15, respectively. It should be emphasized that their values pertain to inter-specific sequence divergences between eight species from four tribes of Cynipids, while our study examined sequence divergence within a single species and between members of a single genus. Accordingly, the inter-specific species sequence divergence of *A. pilosellae* and other species of *Aulacidea* observed in our study was less dramatic, but the sequence divergences between gene regions still differed substantially and followed patterns similar to those observed by Rokas et al. (2002b).

By sequencing *A. subterminalis*, we were able to compare sequence divergence between *A. pilosellae* and what is widely believed to be its most related congener (Nieves-Aldrey 1994,

2001). Interestingly, we found the greatest sequence divergence between *A. pilosellae* and *A. subterminalis* occurred in the ITS2 region (14.9%), although substantial variation still existed in the CO1 region (9.4%) and less in the 28S-D2 region (2.9%). Obtaining the sequences of the four species of *Aulacidea* for which CO1 and 28S-D2 sequences were available in GenBank allowed us to estimate and compare the percent sequence divergence between *A. pilosellae* and all other available species in the genus at both CO1 and 28S-D2. Here, we found the average sequence divergence to be 12.5% and 4.8% respectively.

More broadly, mitochondrial gene regions are generally accepted to diverge more rapidly than nuclear genes (DeSalle et al. 1987, Simon et al. 1994, Moriyama and Powell 1997, Monteiro and Pierce 2001) and a recent meta-analysis estimates mitochondrial gene regions to evolve approximately six times faster than nuclear regions in insects (Lin and Danforth 2004). While the CO1 region is the most widely used region for phylogenetic studies in insects and has become a standardized tool for molecular taxonomy and identification (Ratnasingham and Hebert 2007), the 28S and ITS regions are also widely used. It is becoming standard practice to employ multiple markers in phylogenetic studies that utilize DNA sequencing, as different loci are well-known to evolve at different rates, and our study further emphasizes the necessity of this approach. Effectively the mitochondrial and nuclear gene regions sequenced for *A. pilosellae* would yield different conclusions if analyzed in isolation from each other.

**Splitting of *Aulacidea pilosellae* into multiple lineages based on CO1 data**

Our results clearly show that the CO1 sequences differ substantially among certain members of *Aulacidea pilosellae*. When analyzed with either a relatively simple TrN neighbour-joining (distance based) or the more complex GTR+G (time reversible) nucleotide substitution model, both models split the individual *A. pilosellae* sequences into a minimum of three lineages (Figure 3.1, 3.4). While the intra-lineage variation was on average 1%, the inter-lineage variation was on average 4% (Table 3.4).

A concept central to DNA barcoding is the 'barcoding gap', which provides a benchmark for determining the level of sequence divergence required to delimit species (Hebert et al. 2004, Meyer and Paulay 2005). Hebert and colleagues (e.g. Hebert et al. 2003) originally suggested a benchmark of 2% sequence divergence to delimit species (i.e. under 2% divergence would indicate membership in the same species, greater than 2% would indicate membership in a

different species), and this has subsequently been frequently used to delimit species (Cognato 2006). Hebert et al. 2003 based the 2% threshold on the notion that intra-specific divergences rarely exceed 2% (Hebert et al. 2003 cites Avise 2000, but Cognato 2006 ascertains Avise 2000 did not make this claim), and this has since been replaced with a somewhat more quantitative approach. For this, Hebert et al. (2004) proposed that the 'barcoding gap' should be a threshold ratio of 10:1 inter- to intra-specific CO1 sequence divergence, that is for any taxonomic group, there should be 10 times the sequence divergence between species as there is within species.

When considered in the context of this barcoding gap, our CO1 data for *A. pilosellae* shows there is four times the sequence variation present between lineages of *A. pilosellae* as compared to within lineages of *A. pilosellae*, which would be insufficient for postulating that these lineages are in fact distinct species. However, this 10:1 barcoding gap has received considerable debate in the literature. Wiemers and Fiedler (2007) found nearly 20% overlap in the ranges of intra- and inter-specific sequence divergence in *Agrodiaetus* butterflies, and suggested that the notion of a consistent barcoding gap is a result of insufficient sampling. Meyer and Paulay (2005) voice similar concerns and state that studies that have led to the development of, or that promote the barcoding gap concept (Hebert et al. 2003, 2004, Barrett and Hebert 2005) have suffered from 'greatly under-sampled' intra-specific variation (by only including 1-2 specimens per species) and inter-specific divergence (by conducting geographically restricted or otherwise incomplete sampling). Meier et al. (2008) argue that while the barcoding gap concept has utility, they caution that using mean inter-specific sequence divergence is misleading and overestimates the size of the barcoding gap. They instead suggest that the minimum inter-specific sequence divergence should be used – but they do not specifically comment on whether the mean intra-specific sequence should continue to be used in comparison, or if a barcoding gap of 10:1 is generally applicable using minimum inter-specific divergence. Meyer and Paulay (2005) further argue that the barcoding gap does not delimit closely related species well, especially in taxonomically understudied groups of insects. In light of the conflicting information in the literature, it is difficult to interpret sequence divergence in isolation.

Our results do show there is an average of 14.9% sequence divergence in the CO1 region of all *Aulacidea* species for which sequences were available (Table 3.4), and a minimum of 9.4% sequence divergence between *Aulacidea* species (*A. pilosellae* and *A. subterminalis*). If we do

follow the 10% barcoding gap, our levels of intra-lineage variation for *A. pilosellae* (1%) are in fact one tenth of the estimated minimum inter-specific variation in the sequenced *Aulacidea* (and $1/15^{th}$ if the average inter-specific variation of 14.9% were to be used). Our results would then support that there is a sufficient amount (10%) of variation between; (i) the CO1 delimited intra-lineage variation of the three lineages of *A. pilosellae*, and (ii) the inter-specific variation for all *Aulacidea*. However, when we consider the variation between these three lineages (inter-lineage, Table 3.4), we see that this average variation is 4%, and that even the minimum intra-lineage variation is 3.3%. Therefore we regard the inter-lineage variation in *A. pilosellae* to be somewhere between intra-specific and inter-specific variation. In short, this inter-lineage value is markedly higher than the intra-lineage variation or acceptable intra-specific variation but below the inter-specific variation. This, together with the general lack of sequence divergence in either of the nuclear gene regions, leads us to suggest that lineages of *Aulacidea pilosellae* indeed have begun to diverge, but have not been isolated for a sufficient time to allow for evolution of the nuclear gene regions. Hence we conclude that the weight of evidence is that our lineages of *A. pilosellae* are not divergent enough to represent a cryptic species complex, but that sub-specific cryptic divergence is present.

**Cryptic host-associated or geography-based differentiation?**

Despite there being insufficient evidence to postulate that *A. pilosellae* represents a cryptic species complex, the variation in the CO1 sequences of *A. pilosellae* presents evidence of both host-associated and geographically based differentiation. The original hypothesis at the outset of this study was that there may be genetic differences between populations of *A. pilosellae* from the Southern and Northern Ranges. Once host use data were compiled and we discovered that only one host species was being galled at each site (Chapter 2), we hypothesized that there were possibly multiple cryptic host-associated forms of *A. pilosellae*. Our DNA sequence results provide some support for both hypotheses.

Host-associated basis of differentiation

We found that individuals from the Southern Range (all ex. *P. officinarum*) were in fact of a different lineage than the majority of conspecifics from the Northern Range (Figure 3.4), which cluster in lineage *A. pilosellae* 3. However, our results show that the Southern Range *A. pilosellae* cluster in a lineage (*A. pilosellae* 1) with other *A. pilosellae* from the Northern Range. This forces us to reject the hypothesis that any genetic differentiation in *A. pilosellae* is based

strictly on geography, i.e. that any genetic differentiation between *A. pilosellae* is observed strictly between populations from the Northern and Southern Range. This result suggests that previously observed differences in the physiological host range, number of generations per year and sex-ratios (Grosskopf et al. 2002, 2003, 2004) of *A. pilosellae* collected from *P. officinarum* in the Southern Range vs. *Pilosella* spp. (not including *P. officinarum*) in the Northern Range are not based on geography but on host species use. Grosskopf's studies were conducted with *A. pilosellae* ex. *P. officinarum* from the Southern Range and *A. pilosellae* from at least some of the host species *P. caespitosa, P. glomerata, P. piloselloides,* and *P. cymosa,* from the Northern Range, and did not include any *A. pilosellae* ex. *P. officinarum* from the Northern Range.

All of the *A. pilosellae* that cluster in the lineage *A. pilosellae* 3 are from the *Pilosella* species *P. glomerata, P. caespitosa* and *P. piloselloides,* whereas individuals that cluster in lineage *A. pilosellae* 1 were collected from either *P. officinarum* or *P. floribunda,* indicating some patterns of host-associated differentiation. Perhaps the most interesting result is that the *A. pilosellae* individuals collected from *P. officinarum* in the Southern Range clustered in the same lineage as individuals collected from *P. officinarum* in the Northern Range, as well as the individual collected from *P. floribunda* in the Northern Range. Furthermore, the specimen we call 'JN', from the CO1 sequence obtained from GenBank, clustered in this lineage. Both the *A. pilosellae* collected from *P. officinarum* in the Southern and Northern Range tested positive for the presence of the bacterial endosymbiont *Wolbachia* as well, but the *A. pilosellae* specimen collected from *P. floribunda* did not. Whether the CO1 sequence of the Southern Range *A. pilosellae* was affected by the presence of *Wolbachia* in the population or not, the grouping of these Northern Range *A. pilosellae* from *P. officinarum* and *P. floribunda* with the Southern Range *A. pilosellae* from *P. officinarum* provides strong evidence that the patterns of sequence divergence in *A. pilosellae* are not solely based on geography. Instead, patterns of sequence divergence in *A. pilosellae* appear to be based on host-association and/or *Wolbachia* infection.

Geography-related differentiation

The majority of Northern Range specimens clustered in the lineage *A. pilosellae* 3 (Figure 3.1, 3.4), which itself has two branches. While specimens from four species of *Pilosella* host plants are represented within the lineage *A. pilosellae* 3, the two branches divide specimens based on geography and not host species (Figure 3.1, 3.4); i.e. specimens cluster based on the sample

region from where they were collected: *A. pilosellae* collected from both *P. glomerata* (L2b,L2c) and *P. caespitosa* (L3) in Region C all cluster in one branch, whereas specimens collected from *P. glomerata* (L8) in Region A, and *P. caespitosa* (L6) and *P. piloselloides* (L7) from B cluster in another branch. This indicates that within the lineage *A. pilosellae* 3, found to gall *P. glomerata, P. caespitosa* and *P. piloselloides*, there is more sequence divergence between geographic regions than host species and that there is no genetic basis or differentiation segregating by host plant species.

Additional differentiation

Specimens from two collection sites cluster in an additional lineage, *A. pilosellae* 2, which in the Bayesian analysis is basal to both *A. pilosellae* 1 and *A. pilosellae* 3. One of these is the samples (ES) collected in Spain, reportedly from *P. officinarum*. As we did not collect these specimens ourselves, we cannot definitively verify the host plant identification. *Pilosella officinarum* is certainly among the most widely distributed species of *Pilosella*, and known to be highly variable itself. While it now is recognized as a single species with one sub-species (Bräutigam and Greuter 2007), it has been described to include as many as 624 sub-species (Zahn 1923). Thus it is possible that this Spanish sample was collected from a form of *P. officinarum* that was differentiated from those sampled in central Europe. In addition, this Spanish *A. pilosellae* ex. *P. officinarum* did not test positive for the presence of *Wolbachia*, whereas our specimens of *A. pilosellae* ex. *P. officinarum* from both the Southern and Northern Ranges of our study area did test positive. Possible explanations for the lack of *Wolbachia* in the Spanish *A. pilosellae* ex. *P. officinarum* are discussed below.

One specimen, L2a, presents a bit of a mystery. Collected from what we believe to be the same host species at the same site (C16) as specimens L2b and L2c, the L2a CO1 sequence was deemed in both the NJ and Bayesian analyses to be more similar to the ES sample than the other L2 samples. It should be noted that the intra-lineage sequence variation in this lineage (*A. pilosellae* 2) was 2.5%, more than double that of either of the other lineages, and closer to the inter-lineage variation between lineages 2 and 3 (3.5%). Both the NJ and GTR models found ES and L2a to be more similar to each other than to the other specimens, but with low branch support (66-70%) in both models. Interestingly, the GTR/Bayesian model deemed these specimens to belong to a lineage basal to *A. pilosellae* 1 and 3 (with low branch support), while

the NJ model deemed *A. pilosellae* 1 to be basal to both *A. pilosellae* 2 and 3, also with low branch support. This difference is in part influenced by the consideration of the ITS data, which places L2a (and L2b) as basal to the remaining *A. pilosellae* samples, as they share a single transition with *A. subterminalis*. Site C16 was characterized by what we would deem an unusually high amount of in-site genetic divergence. Both the 28S-D2 sequences (which show the single transition in L2a) and the ITS2 sequences (which show a shared single transition in both L2a and L2b) from specimens at site C16 have higher variability than sequenced specimens from any other site. Site C16 was in fact the only site where any divergence was observed among specimens at a site, and it is very interesting that divergence was seen in all three gene regions, but not consistently between specimens and gene regions. Our interpretation of the divergence in the CO1 sequences of ES and L2a is that there is likely more variation present in *A. pilosellae* than our study detected, and that these two specimens are an indication of greater genetic differentiation in *A. pilosellae*.

**Host-associated detection of *Wolbachia***

Studies conducted by Grosskopf (*unpublished data*) indicated that while *A. pilosellae* ex. *Pilosella* spp. (not *P. officinarum*) from the Northern Range were represented by approximately equal sex ratios, *A. pilosellae* ex. *P. officinarum* from the Southern Range showed an extremely female-biased sex ratio. These *A. pilosellae* ex. *P. officinarum* from the Southern Range were inferred to be parthenogenetic, as female-only populations reproduced successfully for multiple generations (Grosskopf, *unpublished data*). The bacterial endosymbiont *Wolbachia*, among other bacterial endosymbionts, is known to induce parthenogenesis and other reproductive manipulations in Hymenopteran hosts. Thus, we hypothesized that the observed parthenogenesis in *A. pilosellae* ex. *P. officinarum* from the Southern Range may be due to infection with *Wolbachia*. While one specimen per host species per sample site per region was tested for the presence of *Wolbachia*, the only specimens that tested positive were both collected from *P. officinarum*, in the Southern and Northern Ranges respectively (Figure 3.1, 3.4). This infection of *A. pilosellae* ex. *P. officinarum* from the Southern Range with *Wolbachia* supports our hypothesis that *Wolbachia* infection may be related to the observed parthenogenesis; however further study is required to understand the relationship between *Wolbachia* infection, parthenogenesis and host plant use of *A. pilosellae*. Furthermore, life history data for populations of *A. pilosellae* ex. *P. officinarum* from the Northern Range are lacking.

Both of the specimens collected from *P. officinarum* in Spain were also tested, but did not show *Wolbachia* infection, while an additional specimen of *A. pilosellae*, ex. *P. officinarum* from Kent, UK was also recently screened and tested positive for infection with *Wolbachia* (P. Coghlin, *pers. comm.*, sequence data not available at the time of submission). *Wolbachia* absent populations at southern latitudes have been found in other species of Cynipids (i.e. *Diplolepis rosae*) that are otherwise prone to *Wolbachia* infection (Plantard et al. 1998), so it is possible that there is an association between warmer climates and the lack of *Wolbachia* in *A. pilosellae*. Rare males and uninfected females are known from *Wolbachia* infected populations (Plantard et al. 1998), offering another possible explanation for the lack of *Wolbachia* in the Spanish samples.

Whether the failure to detect *Wolbachia* in the ES samples reflects a true absence of *Wolbachia* in this population is unknown. It is unlikely that these specimens contained intact *Wolbachia* DNA, as we used the horn fly *H. irritans* as a positive control for *Wolbachia* in all wsp amplifications. Although infection was not detected it is remotely possible that the relatively small amounts of *Wolbachia* DNA that may have been present at collection did not survive the > 5 years of storage in ethanol. Although we are not aware of data on DNA degradation rates of *Wolbachia* specifically, it is well established that insect specimens stored in ethanol at room temperature for more than one year can experience serious DNA degradation (Carvalho and Vieira 2000). Lastly, samples of the host plant were not available to definitively confirm if it was in fact *P. officinarum*. However, we must assume in light of the lack of any supporting evidence to the contrary, that these Spanish *A. pilosellae* ex. *P. officinarum* were not in fact infected with *Wolbachia*.

Whether *Wolbachia* infection in insects induces lineage disruption through cytoplasmic incompatibility as an isolating mechanism remains debated in the literature. A number of authors have cautioned this may be the case (e.g. Gompert et al. 2008, Nice et al. 2009), but thus far there are no definitive examples of this occurring (Smith et al. 2012). The data presented in this study show that while *A. pilosellae* was collected from multiple host plant species at multiple sites, only *A. pilosellae* collected from *P. officinarum* tested positive for infection with *Wolbachia*. Interestingly, *A. subterminalis*, which is known to exclusively use *P. officinarum* as a host plant, also tested positive for *Wolbachia* and lateral transfer between *A. subterminalis* and *A. pilosellae* ex. *P. officinarum* is possible. While the one *A. pilosellae* specimen from *P.*

*officinarum* that did not test positive for *Wolbachia* (ES) nested in a different lineage than those *A. pilosellae* ex. *P. officinarum* that tested positive (L1 and L4), patterns of association between infection status with *Wolbachia*, host plant association and the life histories of the different biotypes of *A. pilosellae* remain to be fully examined.

**Is *Aulacidea pilosella*e present in North America?**

A CO1 sequence recorded only as Hymenoptera sp. in GenBank was included in our phylogenetic analyses due to the high sequence similarity revealed by the BLASTn of our *A. pilosellae* consensus sequence. This specimen, JN288739, aligned in the *A. pilosellae* 1 lineage, with the individuals collected from *P. officinarum* and *P. floribunda*, and differed from the members of this lineage by only 0.2-1.0%, less than other individuals in different lineages. While several species of *Aulacidea* are known in North America (Beutenmüller 1910), *Aulacidea pilosella*e is not reported. Significantly, *Aulacidea hieracii* was reported on *Hieracium umbellatum* in Sudbury, Ontario in 2003 (Sliva and Shorthouse 2006), and it is likely that it was introduced from Europe as it was not mentioned previously in the North American literature. While we only had one *A. hieracii* CO1 sequence in our analysis (from GenBank), this sequence had at least 14.6% sequence divergence from all the *A. pilosellae* sequenced, so we think it is rather unlikely that JN288739 is *A. hieracii*. Further, the data available in the BOLD database (http://boldsystems.org/index.php/Public_RecordView?processid=ASGLE1501-10) shows the body size of the specimen to be approximately 1.5 mm in size and certainly <2mm in size, while *A. hieracii* is known to be 2.3-2.8mm in size and *A. pilosellae* to be 1.0-1.5mm in size (Dalla Torre and Kieffer 1910). Thus, based on the available evidence, we do not suspect that the specimen JN288739 is *A. hieracii* but rather suspect it is *A. pilosellae*. We have contacted the collector of this specimen and await sequencing of either the 28S-D2 or ITS2 gene regions to aid in confirming the identification of this specimen. If these regions support our hypothesis that this specimen is in fact the first known representative of *A. pilosellae* in North America, we would recommend the specimen be sent to J.L. Nieves-Aldrey, the most appropriate taxonomist, for morphological identification. Further, we would suggest that populations of *Pilosella* hawkweeds be located near the trap location in Guelph, Ontario and that these plants be searched for additional specimens.

**Inferences about the other species of *Aulacidea***

Our results confirm that of the species of *Aulacidea* sequenced, *Aulacidea subterminalis* is the most closely related species to *A. pilosellae*, and these results are consistent across the gene regions sampled. While we only had CO1 and 28S-D2 data available for the other species of *Aulacidea*, these two gene regions show contradictory patterns of relatedness among the *Aulacidea*. The CO1 dataset indicates that *A. pilosellae* and *A. subterminalis* share a more recent common ancestor with *A. freesei* (ex. *Silybum*/*Carduus*, Asteraceae), *A. tragopogonis* (ex. *Tragopogon*, Asteraceae) and *A. phlomica* (*Phlomis*, Lamiaceae) than they do with *A. hieracii* (ex. *Hieracium*, Asteraceae). The 28S-D2 dataset indicates that *A. pilosellae*, *A. subterminalis* and *A. hieracii* all share a more recent common ancestor with each other than they do with the other three species of *Aulacidea*, a more plausible scenario considering that the *Pilosella* and *Hieracium* are closely related genera (Fehrer et al. 2007), and have been previously considered sub-genera within the same genus (*Hieracium*).

While a number of new species of *Aulacidea* have been described in Eurasia in recent years (Nieves-Aldrey 1995, 2004, Meilka 2004, Melika and Gharaei 2006), we are not aware of a taxonomic treatment for the genus *Aulacidea* since Eady and Quinlan (1964). In earlier iterations of the CO1 NJ tree in this study, we included sequences obtained from the BLASTn that were highly similar, and observed that both *Iraella luteipes* (Cynipidae: Aylacini, ex. *Papaver*, Papaveraceae) and *Neaylax versicolor* (Cynipidae: Aylacini, ex. *Fumaria*, Fumariaceae) both nested within the species of *Aulacidea* in our tree (data not shown). Considering there are at least 27 species of Palaearctic *Aulacidea* (Nieves-Aldrey 2004, Melika and Gharaei 2006) and more still in the Nearctic, we suggest a taxonomic revision of the *Aulacidea* is overdue, and urge the holders of specimens of other species of *Aulacidea* to have them sequenced at both CO1 and a nuclear gene region and data added to GenBank.

**Summary and future directions**

Our study is the first to test the hypothesis that there may be cryptic genetic divergence between the two purported biotypes of *Aulacidea pilosellae*. This work presents a number of significant findings; genetic divergence does exist between populations of *A. pilosellae*, but these patterns are not solely based on geography or host plant association. Instead, the patterns of divergence revealed in our study indicate that *A. pilosellae* ex. *P. officinarum* are largely distinct from *A.*

*pilosellae* from other *Pilosella* host plants, e.g. *P. glomerata, P. caespitosa* and *P. piloselloides* (but not *P. floribunda*), but not because of geography as was previously proposed. Our finding of *A. pilosellae* on *P. officinarum* in the Northern Range was significant – and demonstrated that the *A. pilosellae* ex. *P. officinarum* from the Southern Range were distinct from *A. pilosellae* ex. *Pilosella* spp. in the Northern Range not because of geographic separation, but due to host plant-association and/or infection with *Wolbachia*. Further, we find that *A. pilosellae* ex. *Pilosella* spp. from the Northern Range are also somewhat diverged –not based on host species – but based on geography. A surprising additional finding of our work was discovering that a sequence highly similar to *A. pilosellae* was already present in GenBank – and that the specimen had been collected in North America, where *A. pilosellae* has not been recorded. While this work regarding the genetic differentiation of *A. pilosellae* presents a number of interesting findings, much more work is needed to resolve patterns of genetic diversity and how it relates to geography of populations, host-plant associations, *Wolbachia* infection, and possibly other factors. Further work could include both observational and experimental population level studies to further clarify if host-associations of *A. pilosellae* are fixed and what role *Wolbachia* infection may play in host use of *A. pilosellae*. More specific suggestions and the implications of these findings to the *Pilosella* biological control programme are discussed in Chapter 4.

**Table 3.1:** Specimen information for the *Aulacidea pilosellae* and *A. subterminalis* used in this study, including the Range (Southern = S, Northern =N), Region, Site ID (for site data see Table 2.1), specimen ID, number of specimens sequenced (N), host plant species and collection location.

| Range | Region | Site ID | Specimen ID | N | *Aulacidea* species | *Pilosella* host plant species | Collection location |
|---|---|---|---|---|---|---|---|
| S | S | S13 | L1 (a,b) | 2 | *A. pilosellae* | *P. officinarum* | Jura Canton, Switzerland |
| N | C | C16 | L2a | 1 | *A. pilosellae* | *P. glomerata* | Velka Upa, Czech Republic |
| N | C | C16 | L2b | 1 | *A. pilosellae* | *P. glomerata* | Velka Upa, Czech Republic |
| N | C | C16 | L2c | 1 | *A. pilosellae* | *P. glomerata* | Velka Upa, Czech Republic |
| N | C | C4 | L3 (a,b) | 2 | *A. pilosellae* | *P. caespitosa* | Milkow, Poland |
| N | C | C1 | L4 (a,b) | 2 | *A. pilosellae* | *P. officinarum* | Gorlitz, Germany |
| N | B | B8 | L5 | 1 | *A. pilosellae* | *P. floribunda* | Reitzanhain, Germany |
| N | B | B7 | L6 (a,b) | 2 | *A. pilosellae* | *P. caespitosa* | Schlettau, Germany |
| N | B | B1 | L7 (a,b) | 2 | *A. pilosellae* | *P. piloselloides* | Dolní Žďár, Czech Republic |
| N | A | A5 | L8 | 1 | *A. pilosellae* | *P. glomerata* | Klingenbrunn, Germany |
| - | | - | ES (a,b) | 2 | *A. pilosellae* | *P. officinarum* | Province of Madrid, Spain |
| S | S | - | Asub (a,b) | 2 | *A. subterminalis* | *P. officinarum* | Jura Canton, Switzerland |

**Table 3.2:** Polymerase Chain Reaction (PCR) thermocycle programs for the three gene regions amplified for *A. pilosellae* and *A. subterminalis*, following the protocols of CO1: *DNA Barcoding Animal Life Using CO1* (dnabarcoding.ca/primer/PDFs/Protocols/Barcodes_of_Life_Laboratory_Protocols_CO1_Amplification.pdf), 28S-D2: Babcock and Heraty (2000), ITS2: Campbell et al. (1993).

| | PCR Program CO1 | | PCR Program 28S-D2 | | PCR Program ITS2 | |
|---|---|---|---|---|---|---|
| Step | Action | Time | Action | Time | Action | Time |
| 1 | 94°C | 1 min | 94°C | 3 min | 97°C | 1 min |
| 2 | 94°C | 30 sec | 94°C | 45 sec | 94°C | 1 min |
| 3 | 45°C | 1 min, 30 sec | 55°C | 30 sec | 50°C | 1 min |
| 4 | 72°C | 1 min | 72°C | 1 min 30 sec | 72°C | 2 min |
| 5 | go to step 2 | 5 more times | go to step 2 | 29 more times | go to step 2 | 28 more times |
| 6 | 94°C | 30 sec | 72°C | 30 min | 72°C | 7 min |
| 7 | 51°C | 1 min, 30 sec | 4°C | 0:00:00 | 4°C | 0:00:00 |
| 8 | 72°C | 1 min | | | | |
| 9 | go to step 6 | 35 more times | | | | |
| 10 | 72°C | 5 min | | | | |
| 11 | 4°C | 0:00:00 | | | | |

**Table 3.3:** Cynipidae sequences used in the phylogenetic analyses; all sequences obtained from GenBank. All species of *Aulacidea* and *D. rosae* were obtained as out-groups, whereas the *Hymenoptera* sp. (accession no. JN288739) was identified from a BLASTn.

| | GenBank Accession No. | | | |
|---|---|---|---|---|
| Cynipid Species | CO1 | 28S | ITS2 | Collection location |
| *Hymenoptera* sp. | JN288739 | - | - | Guelph, Ontario |
| *Aulacidea hieracii* | DQ012628 | DQ012586 | - | Europe |
| *Aulacidea tragopogonis* | AY368922 | AY368948 | - | Europe |
| *Aulacidea freesei* | DQ012627 | DQ012585 | - | Europe |
| *Aulacidea phlomica* | DQ012629 | DQ012587 | - | Europe |
| *Diplolepis rosae* | AF395174 | AF395157 | JN252389- | Europe |

**Table 3.4:** Intra- and inter-specific percent sequence divergence (nucleotide divergence), standard deviation (range) of the concatenated three gene region dataset (CO1-28S-ITS2), the concatenated two gene region dataset (CO1-28S), and each gene region separately. All datasets contain 19 sequences of *Aulacidea pilosellae*, 2 of *A. subterminalis*, one each of four additional *Aulacidea* species: *A. hieracii*, *A. tragopogonis*, *A. freesei*, *A. pholmica*, and *Diplolepis rosae* except the CO1-28S-ITS and ITS datasets which do not contain the four additional *Aulacidea* species. The *Hymenoptera* sp. (accession no. JN288739) CO1 sequence is present in all datasets using CO1 data.

| | **CO1-28S-ITS2 mean, SD (range)** | **CO1-28S mean, SD (range)** | **CO1 mean, SD (range)** | **28S mean, SD (range)** | **ITS2 mean, SD (range)** |
|---|---|---|---|---|---|
| **Intra-specific** | | | | | |
| **Intra-lineage** | | | | | |
| *A. pilosellae* 1 | 0.4, 0 (0.4-0.4) | 0.2, 0 (0.2-0.2) | 0.55, 0.36 (0.2-1.0) | 0.0, 0 (0.0) | 0.4, 0 (0.4-0.4) |
| *A. pilosellae* 2 | 1.1, 0 (1.1-1.1) | 2.0, 0 (2.0) | 2.5, 0 (2.5-2.5) | 0.2, 0 (0.2) | 0.4, 0 (0.4-0.4) |
| *A. pilosellae* 3 | 0.71, 0.4 (0.1-1.1) | 0.4, 0.4 (0.0-1.1) | 1.1, 0.7 (0.0-2.0) | 0.0, 0 (0.0) | 0.6, 0.4 (0.0-1.3) |
| Average | 0.72, 0.4 (0.1-1.1) | 0.5, 0.6 (0.0-2.0) | 1.0, 0.7 (0.0-2.5) | 0.01, 0.05 (0.0-0.2) | 0.58, 0.4 (0.0-1.3) |
| **Inter-lineage** | | | | | |
| *A. pilosellae* 1–2 | 1.6, 0.2 (1.5-1.8) | 2.2, 0 (2.2) | 4.0, 0.2 (3.7-4.3) | 0.1, 0.1 (0.0-0.2) | 0.33, 0.2 (0.2-0.6) |
| *A. pilosellae* 1–3 | 1.9, 0.3 (1.6-2.3) | 2.3, 0.1 (2.2-2.5) | 4.3, 0.4 (3.9-5.0) | 0.0, 0 (0.0) | 0.49, 0.5 (0.0-1.3) |
| *A. pilosellae* 2–3 | 1.6, 0.2 (1.3-1.9) | 2.0, 0.3 (1.5-2.2) | 3.5, 0.2 (3.3-3.9) | 0.1, 0.1 (0.0-0.2) | 0.43, 0.4 (0.0-1.1) |
| Average | 1.8, 0.3 (1.3-2.3) | 2.2, 0.2 (1.5-2.5) | 4.0, 0.5 (3.3-5.0) | 0.06, 0.09 (0.0-0.2) | 0.44, 0.4 (0.0-1.3) |
| **Inter-specific** | | | | | |
| *A. pil – A. subter.* | 9.1, 0.2 (8.8-9.5) | 6.5, 0.2 (6.2-6.8) | 9.4, 0.4 (8.9-10.0) | 2.9, 0.06 (2.9-3.1) | 14.9, 0.2 (14.7-15.3) |
| *A. pil – A. hieracii* | NA | 10.4, 0.3 (9.8-10.8) | 15.5, 0.6 (14.6-16.4) | 3.8, 0.06 (3.8-4.0) | NA |
| *A. pil – Aulacidea* | NA | 9.0, 1.4 (6.2-10.8) | 12.5, 2.3 (8.9-16.4) | 4.8, 1.7 (2.9-7.8) | NA |
| All *Aulacidea* | NA | 11.0, 1.4 (9.3-13.9) | 14.9, 2.6 (10.9-18.3) | 6.2, 2.1 (2.3-8.9) | NA |

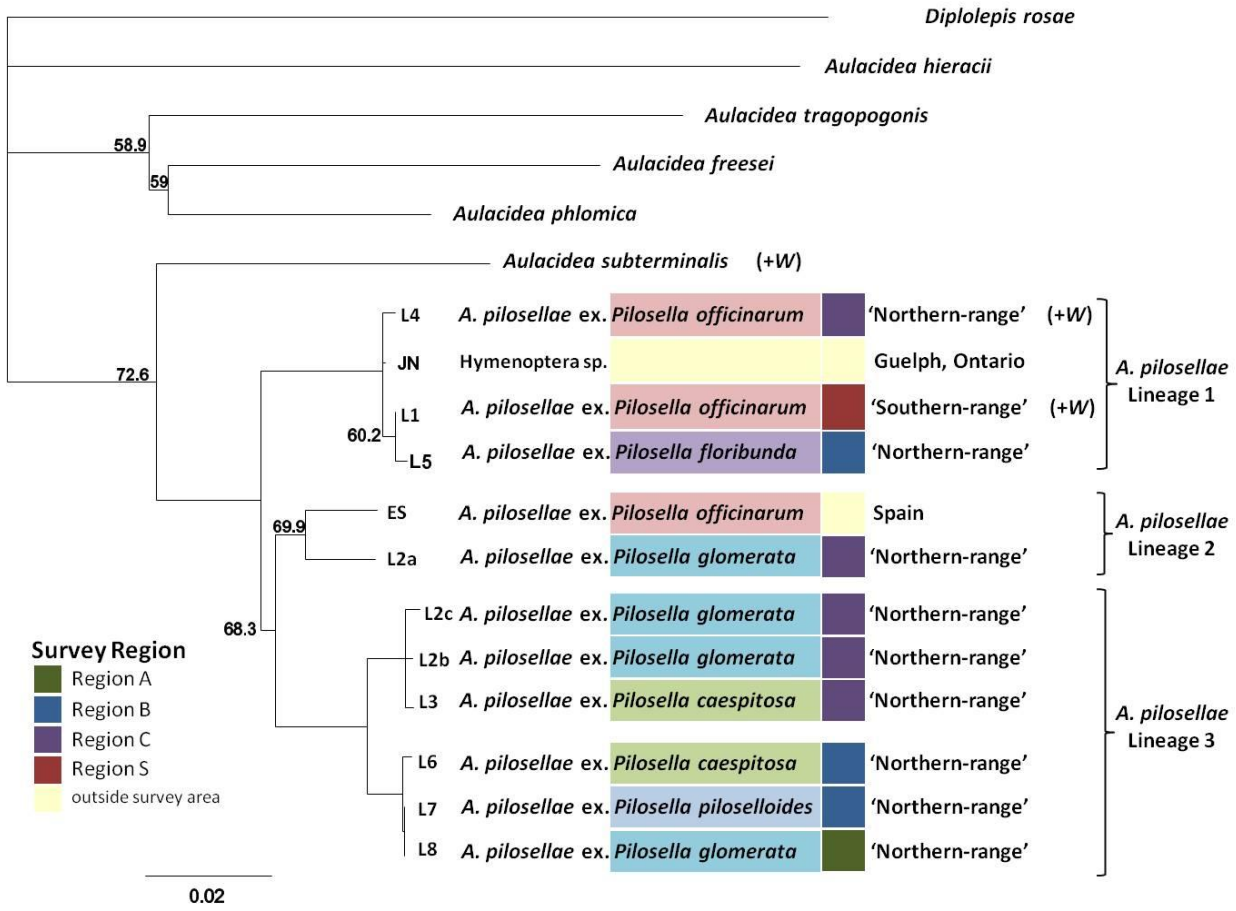


**Figure 3.1:** Tamura-Nei neighbour joining tree of genetic distance for the CO1 mtDNA gene region, of *Aulacidea pilosellae* (L1-L8 this study, ES Spain), an unidentified CO1 sequence from a specimen collected in a Malaise trap near Guelph, Ontario (JN), all other known sequenced species of *Aulacidea*: *A. subterminalis* (this study), *A. freesei*, *A. phlomica*, *A. hieracii*, and *A. tragopogonis* (all from GenBank) and a tribe-level outgroup, *D. rosae* (Genbank). Coloured rectangles high-lighted host species while coloured squares denote the survey region from which the specimen was collected. Accession numbers for sequences obtained from GenBank are included in Table 3.3. All samples sequenced as part of this study (*A. pilosellae* and *A. subterminalis*) were tested for the endosymbiont *Wolbachia*, specimens that tested positive are denoted as (+*W*).

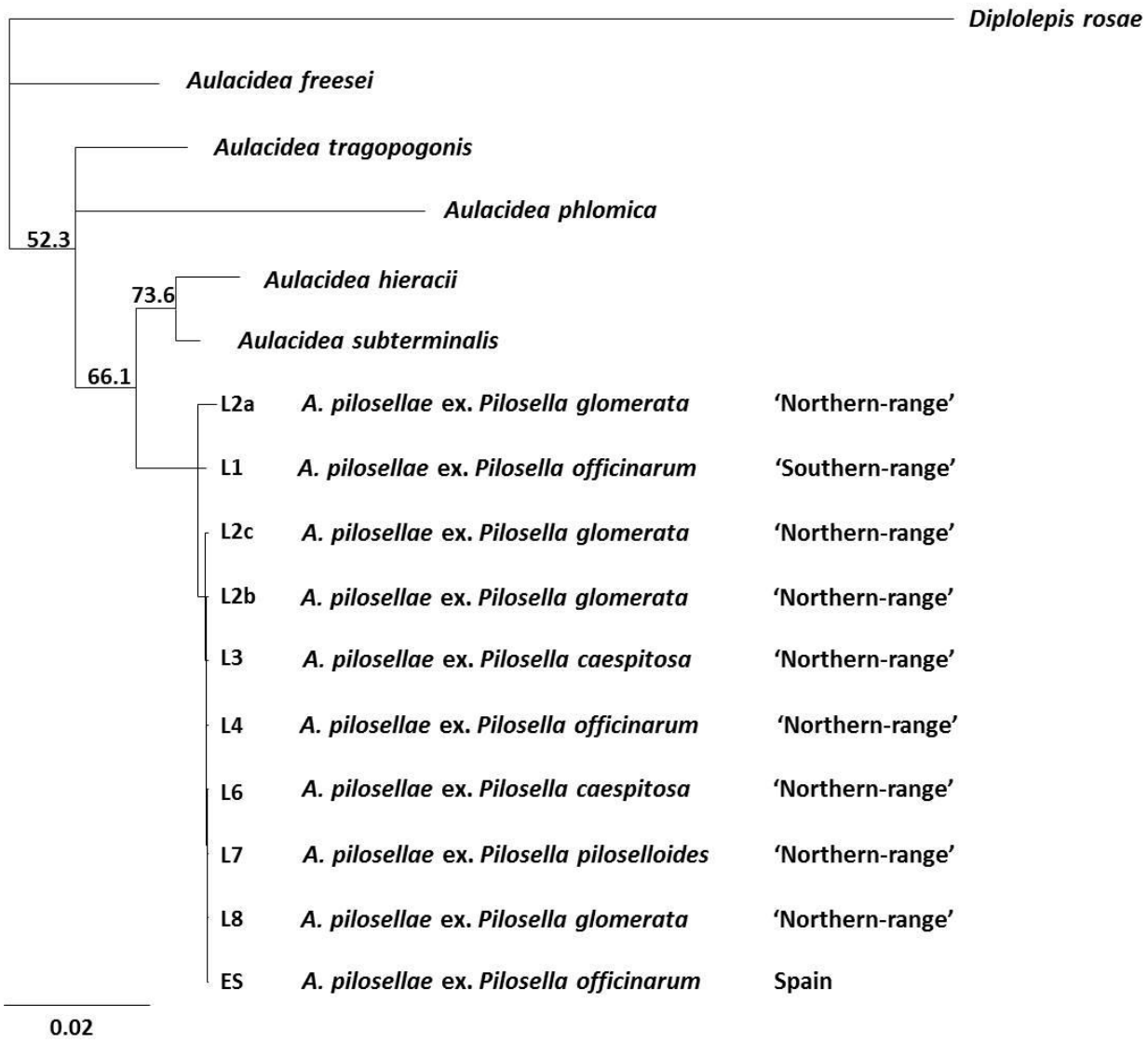


**Figure 3.2:** Tamura-Nei neighbour joining tree of genetic distance for the 28S-D2 rDNA gene region of *Aulacidea pilosellae* (L1-L8, this study and ES, Spain), all other known sequenced species of *Aulacidea*: *A. subterminalis* (this study), *A. freesei*, *A. phlomica*, *A. hieracii*, and *A. tragopogonis* (all from GenBank) and a tribe-level outgroup, *D. rosae* (Genbank). Accession numbers for sequences obtained from GenBank are included in Table 3.3.

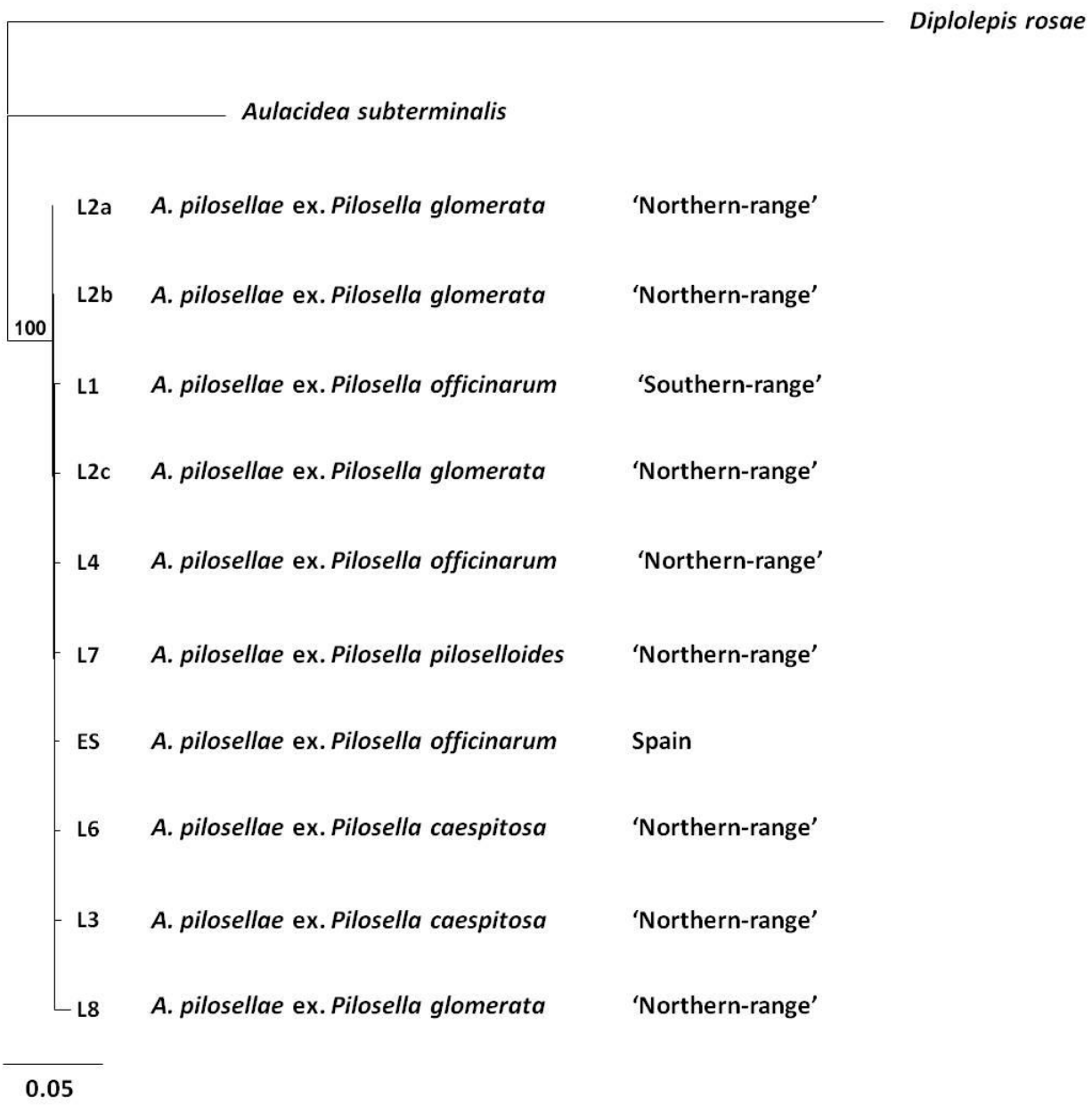


**Figure 3.3:** Tamura-Nei neighbour joining tree of genetic distance for the ITS2 rDNA gene region of *Aulacidea pilosellae* (L1-L8, this study and ES, Spain), *Aulacidea subterminalis* (this study), and a tribe-level outgroup, *D. rosae* (Genbank). Accession numbers for sequences obtained from GenBank are included in Table 3.3.

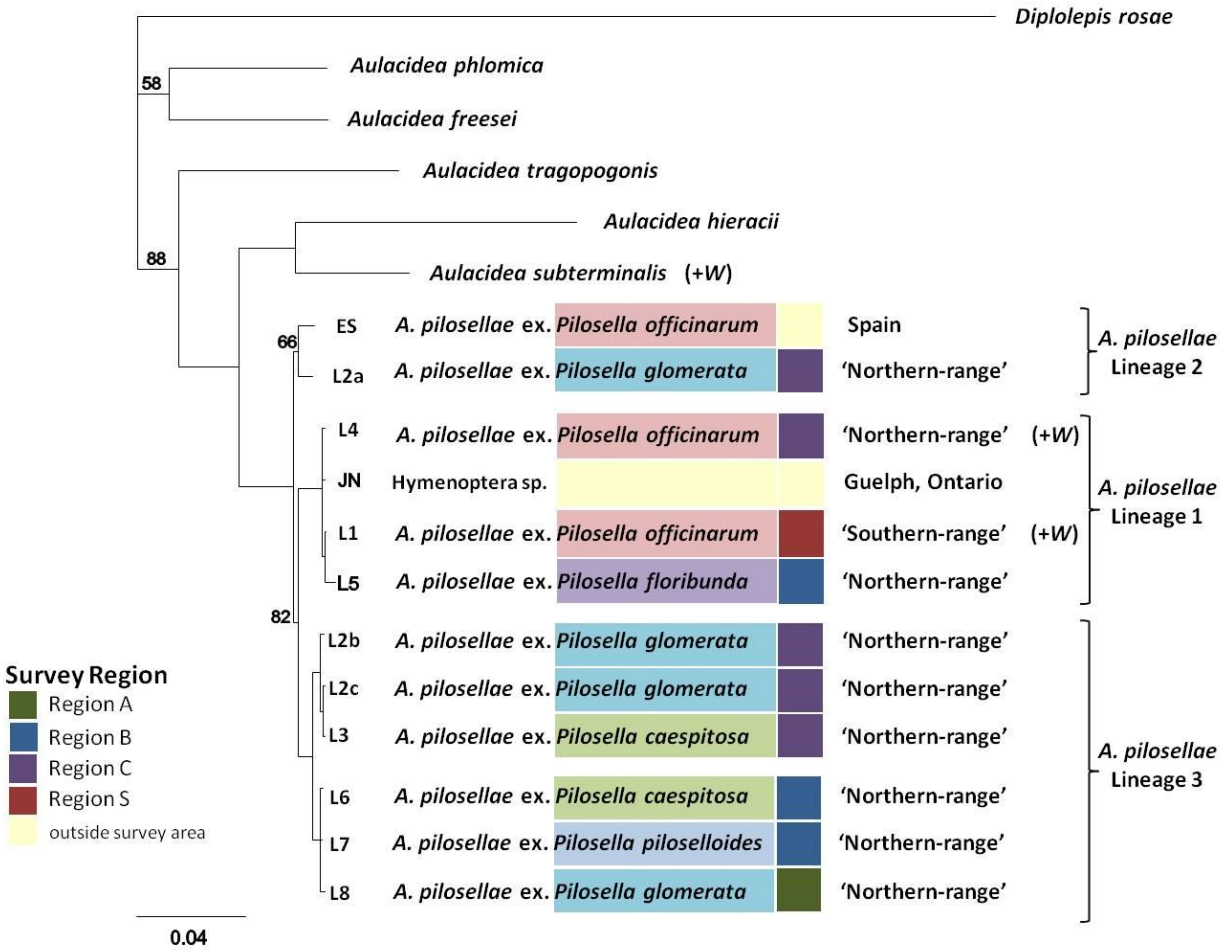


**Figure 3.4:** MrBAYES 50% majority-rule consensus phylolgeny for the concatenated CO1-28SD2-ITS2 three-gene region dataset of *Aulacidea pilosellae* (L1-L8, this study and ES, Spain), an unidentified CO1 sequence from a specimen collected in a Malaise trap near Guelph, Ontario (JN), all other known sequenced species of *Aulacidea*: *A. subterminalis* (this study), *A. freesei*, *A. phlomica*, *A. hieracii*, and *A. tragopogonis* (all from GenBank) and a tribe-level outgroup, *D. rosae* (Genbank). Phylogeny constructed assuming a combined GTR+G (CO1) / HKY (28SD2) / F81 (ITS2) strict clock model of sequence evolution. Numbers by nodes denote posterior probabilities (expressed as a percent), any unlabelled nodes have a posterior probability of ≥99.87%. Notation ex. is followed by the host plant species from which the specimen was obtained. Coloured rectangles high-lighted host species while coloured squares denote the survey region from which the specimen was collected. Accession numbers for sequences obtained from GenBank are included in Table 3.3. All samples sequenced as part of this study (*A. pilosellae* and *A. subterminalis*) were tested for the endosymbiont *Wolbachia*, specimens that tested positive are denoted as (+*W*). Two specimens were sequenced per sample of A. pilosellae where possible. JN and L5 are only represented by CO1 data while A. freesei, A. phlomica, A. hieracii, and *A. tragopogonis* are represented by CO1 and 28SD2 data, missing data was encoded as null.

# **CHAPTER 4:** IMPLICATIONS OF THE PATTERNS OF HOST USE AND CRYPTIC DIVERGENCE OF *AULACIDEA PILOSELLAE* FOR THE *PILOSELLA* BIOLOGICAL CONTROL PROGRAMME


## SUMMARY

Several species of *Pilosella* hawkweeds of European origin are invasive in North America. These *Pilosella* hawkweeds are known to have a number of detrimental environmental impacts (Lass and Callihan 1997, Wilson et al. 2006) and are estimated to have eventual economic impacts in the tens of millions of dollars (Frid et al. 2009). While the members of this taxonomically difficult genus are often difficult to distinguish morphologically and grow in sympatry, their specialized natural enemies readily discriminate between species (Grosskopf et al. 2008). Members of this genus have been targeted for biological control since the 1990s, however only one agent has been approved for release and its ecological host range in its native Europe is only known to be a single species, *Pilosella officinarum*. At least seven other species of *Pilosella* in western North America remain without biological control agents and continue to spread. A promising candidate agent, the gall wasp *Aulacidea pilosellae* Kieffer, is reported from a number of *Pilosella* species, but its patterns of host use in previous biocontrol related studies were inconclusive.

The aims of this thesis were to; (i) determine if *A. pilosellae* exhibits conclusive patterns of host-preference among species of *Pilosella* within its ecological host range, by conducting systematic and wide-spread surveys across the known European distribution of *A. pilosellae*, (ii) further quantify the ecological host range of *A. pilosellae* across its native European distribution, and (iii) determine if there is a genetic basis of differentiation between the two purported biotypes of *A. pilosellae*, and if such genetic divergence occurs, if it is substantial enough to warrant considering that *A. pilosellae* may represent a cryptic species complex. The original intention of this work was to assess patterns of host plant species use in the European native range of *A. pilosellae*, in an effort to provide information to aid in predicting which species of *Pilosella* would most likely be utilized by *A. pilosellae* if approved for release in North America. This thesis advances the knowledge of the patterns of host species use by *A. pilosellae* in its native range, and provides suggestions for future study and best practices.

Our study is among the first in the field of weed biological control to integrate systematic ecological assessments of patterns of host species across the broad distribution of a candidate weed biocontrol agent in its native range, with the use of molecular-based approaches, prior to introduction. Our results show, first and foremost, that the interpretations of either portion of the study in isolation would not yield the same conclusions as our integrative approach. Specifically, the patterns of use of *Pilosella officinarum* by *A. pilosellae* shown in the ecological host use study (Chapter 2), which in part obscured the observed patterns of frequency-dependent host use, can be explained by the patterns of genetic differentiation shown in the cryptic diversity study (Chapter 3). Secondly, the patterns of cryptic diversity are arguably only of biological relevance when examined in the context of host plant association and the geographic range of collection. Thirdly, we found additional benefits of such an approach by further incorporating molecular approaches – the parthenogenesis observed by Grosskopf led to testing for the presence of *Wolbachia*, which may play a role in the divergence of the *A. pilosellae* ex. *P. officinarum* lineage. Further, by searching our *A. pilosellae* sequence data in publicly available databases we found that the insect may already be present in North America. All of these findings have strong implications for the *A. pilosellae – Pilosella* biological control programme and potential applications to the field of weed biological control.

## FREQUENCY-DEPENDENT HOST USE

### Patterns of frequency-dependent host use in consideration of cryptic divergence

In Chapter 2, we hypothesized that *A. pilosellae* would show definitive preferences for species of *Pilosella* with its ecological host range, and predicted that the gall wasp would strongly prefer *P. officinarum* (due to its common mention as a host in the literature). We also predicted *A. pilosellae* would exhibit preferences for *P. glomerata* and *P. caespitosa* (as Grosskopf found these species commonly galled). Our findings show no support for this hypothesis and its associated predictions, and we instead show that wherever *A. pilosellae* is presented with a 'choice' of putative host species, only one species is used. When we analyzed these data, we considered all species of *Pilosella* we found used by *A. pilosellae* across the study equally, thus we included *P. officinarum* as a host in the 'preference' (frequency-dependence) analysis. The results of Chapter 3 demonstrate that the *A. pilosellae* ex. *Pilosella* spp. in the Northern Range (lineage *A. pilosellae* 3) is genetically divergent from *A. pilosellae* ex. *P. officinarum* (lineage *A. pilosellae* 1) regardless of Range (Figure 3.1, 3.4). In combination with Grosskopf's findings

that the *A. pilosellae* she collected from *Pilosella* spp. in the Northern Range did not accept *P. officinarum* as a host in testing of the physiological (no-choice) host range, we suggest there is sufficient evidence to conclude that *A. pilosellae* ex. *P. officinarum* (lineage *A. pilosellae* 1) and *A. pilosellae* ex. *Pilosella* spp. (lineage *A. pilosellae* 3) have divergent ecological and physiological host ranges. In light of these results, we present a second version of our frequency-dependent host selection analysis, this time without including *P. officinarum* as a putative host species at sites where other species of *Pilosella* were utilized. While this decreases our sample size to only four sites where *A. pilosellae* ex. *Pilosella* spp. had a 'choice' between multiple putative host species, at all four sites the most abundant putative host species was the only species utilized as a host. Our re-analysis of this data, again using a one-tailed Fisher's exact test, shows that our finding that the most abundant putative host *Pilosella* species was galled by *A. pilosellae* significantly more often than expected by chance ($P = 0.014$, odds ratio = $\infty$) still holds.

**Implications of frequency-dependent host species selection to the biocontrol programme**

The implications of this pattern of frequency-dependent attack to the *A. pilosellae-Pilosella* biocontrol programme may vary depending on the mechanism regulating this pattern. We suggest, in Chapter 2, that possible processes behind the frequency-dependency pattern could include local adaptation/host tracking of *A. pilosellae* to the most common host (Kaltz and Shykoff 1998, Lively and Dybdahl 2000) or 'predator' switching (Cornell 1976). As discussed in Chapter 2, if host tracking is the mechanism for frequency-dependent host use by *A. pilosellae*, then the number of generations required for *A. pilosellae* to effectively track the most abundant host in a novel habitat could have important implications for the biocontrol programme. If *A. pilosellae* took several generations to host track once released in mixed populations of *Pilosella* in the introduced range, perhaps host use would be dispersed over multiple *Pilosella* species. Alternatively, if *A. pilosellae* began host tracking rapidly, then likely only the most abundant available *Pilosella* species would be used. Whether *A. pilosellae* would then switch host species if it were enacting effective control over the attacked host species (and its abundance became lower than that of a congener), or become locally extinct, would again have different implications for the biological control programme. This (host-switching) would be effectively the same as the predator switching mechanism, which if supported would suggest that *A. pilosellae* is a 'switching generalist' and would gall the most abundant host in any new habitat. Under either of these two scenarios, if *A. pilosellae* did transition from one species to

another, locally depending on which species was the most abundant, this would potentially be a beneficial strategy of sequentially controlling multiple *Pilosella* species at a site. These scenarios remain purely speculative based on available data, but in light of the different outcomes these scenarios could have for the biological control programme, they may merit further research.

**Limitations and suggestions for future work to assess patterns of frequency-dependence**

Especially when we remove *P. officinarum* from the frequency-dependent analysis, our inferences are based on very low sample sizes (n=4). As the patterns of host species selection by *A. pilosellae* ex. *Pilosella* spp. followed a strictly binomial distribution in our study, the pattern was significant despite these small sample sizes. Prior to our data regarding frequency-dependent host species selection of *A. pilosellae* ex. *Pilosella* spp. being considered 'definitive' for this type, we would strongly suggest further study. While an increase in observational sample size number (i.e. more field sites where multiple species of putative *Pilosella* hosts are present in sympatry with *A. pilosellae*) would be ideal, we doubt the feasibility of such a quest. Our study systematically surveyed 56 sites of *Pilosella* hawkweeds across four countries in central Europe, 39 of these in the Northern Range. Further, we performed dozens more ad-hoc searches in many small roadside patches of varying *Pilosella* species we deemed insufficiently small for full surveys, where we did not find *A. pilosellae* (data not shown). Across all of these sample sites and ad-hoc searches, we only documented nine sites where *A. pilosellae* was found with multiple putative *Pilosella* host species, and when *P. officinarum* was excluded, only four. Thus, while our own study may suffer from small sample sizes, urging further work to increase sample size in such a study is from our perspective, lacking feasibility. We would instead suggest that a feasible (although possibly cumbersome) way to further test if patterns of frequency-dependent host use are the norm for *A. pilosellae*, and to begin to assess the mechanism behind such a pattern, would be through a combination of careful field collections (keeping track of the relative abundance of putative *Pilosella* host species and from which species *A. pilosellae* was collected), experimentation in replicate multi-*Pilosella* species communities, and ecological modelling. As is acknowledged in Chapter 2, this type of study far exceeds the aims and resources of biological control programmes, but may be of empirical value.

## CRYPTIC DIVERGENCE

### Patterns of cryptic divergence in consideration of host use data

A number of recent studies demonstrate the utility of combining DNA sequence data with ecological observations (e.g. Smith et al. 2008, Tan et al. 2010), which in the case of specialist insects, is especially important, as is using multiple markers in DNA sequencing studies (Rubinoff and Holland 2005). Our study clearly benefits from this approach, by using ecological data to inform our molecular study which employed multiple markers, and in turn, interpreting the results of our molecular study in consideration of the ecological data.

The careful ecological and physiological host use data, as well as life history observations of *A. pilosellae*, compiled by Grosskopf (2002, 2003, 2004), led to the hypothesis that there were two biotypes of *A. pilosellae* based on geographic distance. Upon reviewing the Grosskopf's original records and our own host use data presented in Chapter 2, we hypothesized that observed differences in the host range and life history may be due to cryptic genetic divergence of *A. pilosellae*, and further hypothesized there was the potential for multiple cryptic species. We specifically predicted (following Grosskopf) that any genetic divergence between the Southern and Northern Range populations would be based solely on geographic segregation, but that any genetic divergence within the Northern Range populatiosn would be based on host species association.

While we found evidence of both host-associated and geography-based cryptic divergence in *A. pilosellae*, our results are contrary to what we predicted. Instead of finding that genetic divergence between the Northern Range and Southern Range could be assumed to be based on geography (i.e. allopatric divergence), we found that it was host-associated. Specifically, we found that *A. pilosellae* ex. *P. officinarum* from both the Southern and Northern Ranges share a CO1 sequence that is much more similar to each other's than *A. pilosellae* ex. *P. officinarum* does with *A. pilosellae* ex. *P. glomerata*, *P. caespitosa*, or *P. piloselloides*, from sites much nearer (Figures 2.1, 2.2, 3.1, 3.4). To our knowledge, Grosskopf never encountered *A. pilosellae* galls on *P. officinarum* in the Northern Range. However, our detection of this one population proved pivotal to interpreting the cryptic variation present in *A. pilosellae*. While we found CO1 sequence divergence between individual *A. pilosellae* sufficient enough to produce multiple

lineages under different models of nucleotide substitution, we did not find sufficient divergence in either of the nuclear gene regions tested. A number of studies make use of only CO1 data to delimit species (e.g. Hebert et al. 2003), however, this practice is becomingly increasingly replaced by studies that employ multiple markers (e.g. Smith et al. 2008). Studies like ours exemplify the necessity of this approach. Had we only sequenced a nuclear gene region, we would have concluded there was very little and no pattern to the genetic divergence seen. Alternatively, if we had only sequenced the CO1 (or another mtDNA region), we may have argued that despite lower than a 10:1 ratio of inter- to intra-specific divergence, that *A. pilosellae* was a cryptic species complex. Instead, we considered data from multiple gene regions, and the lack of divergence in the nuclear regions likely has tempered our excitement over finding divergence in the CO1 region.

Perhaps of the greatest relevance, in both basic and applied regards, is how our interpretations of the sequence data change when considered with the host use and life history data. In the absence of the host range and life history (voltinism, reproductive mode) data, the disagreements in gene region sequence divergence could have been interpreted as aberrant mtDNA mutations (Dasmahapatra et al. 2009). Instead, when combined especially with the physiological host range testing conducted by Grosskopf et al. (2004) that showed that *A. pilosellae* ex. *P. officinarum* (from the Southern Range) did not accept *P. glomerata* or *P. caespitosa* as hosts while *A. pilosellae* ex. *Pilosella* spp. (from the Northern Range) accepted these species but not *P. officinarum*, we believe there is sufficient combined ecological-molecular data to support the hypothesis that there is true host-associated divergence in *A. pilosellae* and to warrant considering *A. pilosellae* to be composed of at least two sub-specific types.

**Implications of cryptic divergence to the biocontrol programme**

The results of the cryptic diversity study have clear implications for the *A. pilosellae-Pilosella* biocontrol programme. Our results confirm that in its native, European range, *Aulacidea pilosellae* uses a number of *Pilosella* hawkweeds as host species, including *Pilosella caespitosa*, *P. glomerata*, *P. floribunda*, *P. officinarum*, *P. piloselloides*, all of which are widely distributed in at least parts of, if not across much of North America (Wilson et al. 2006, Wilson 2006 revised 2007). We further find what we deem to be sufficient evidence to consider that there are two sub-specific types of *A. pilosellae*, which we refer to as *A. pilosellae* ex. *P. officinarum* and

*A. pilosellae* ex. *Pilosella* spp., and that these types have divergent ecological (yet somewhat overlapping physiological) host ranges.

Based on our findings regarding patterns of host use and cryptic diversity of *A. pilosellae*, we strongly suggest that *A. pilosellae* ex. *P. officinarum* in the Southern Range only be considered as a candidate biocontrol agent for *Pilosella officinarum*, although testing the levels of acceptance and galling incidence on *P. floribunda* may also be valuable. We further suggest that any *A. pilosellae* collected on *P. officinarum* or *P. floribunda* in the Northern Range be kept separate from any *A. pilosellae* collected from *P. glomerata*, *P. caespitosa* and *P. piloselloides*, and its physiological host range assessed separately. While *A. pilosellae* collected from *P. officinarum* or *P. floribunda* in the Northern Range aligned in the same lineage as *A. pilosellae* ex. *P. officinarum* from the Southern Range, we should not assume that they will also share physiological host ranges and life histories with *A. pilosellae* from the Southern Range.

*Pilosella caespitosa* is one of the most widespread and noxious of the invasive *Pilosella* species in western North America (Gaskin and Wilson 2007). Its putative hybrid offspring, *P. glomerata*, is also emerging as a serious invader (Ensing et al. *in prep.*). However, both species lack any approved biocontrol agents. We thus suggest that any efforts in the *Pilosella* biological control programme regarding *A. pilosellae* focus on further evaluating this candidate agent for these species. We suggest that *A. pilosellae* ex. *Pilosella* spp. (lineage *A. pilosellae 3*), especially collected from *P. glomerata* and *P. caespitosa* in Region C of the Northern Range where *A. pilosellae* and use of these species was most common, be utilized as candidate agents for *P. glomerata* and *P. caespitosa*. Based on the available data (our ecological and molecular study, Grosskopf's physiological host range data) we do not expect there to be further differences in host use between wasps collected from these two *Pilosella* species, especially in Region C. Despite our *A. pilosellae* ex. *P. piloselloides* aligning in the same lineage as those collected from *P. glomerata* and *P. caespitosa*, we hesitate to suggest any further *A. pilosellae* ex. *P. piloselloides* be pooled with wasps collected from *P. glomerata* and *P. caespitosa*. As stated in earlier chapters, we were not able to definitively confirm identification of this species and only specimens from a single galled plant were available for inclusion in the phylogenetics analysis. As such, we caution that any *A. pilosellae* ex. *P. piloselloides* should be tested separately. We suggest that populations of *A. pilosellae* ex. *P. glomerata* and *P. caespitosa*

could be tested as control agents for other species of *Pilosella* invasive in North America as it is possible this lineage/type accepts more *Pilosella* species than we quantified in our study. Possible species to test include the *P. piloselloides* complex (i.e. *P. piloselloides* and *P. piloselloides* subsp. *praealta*), and other species reported as hosts in the literature (i.e. *P. flagellaris*) but not encountered in our surveys.

Finally, we do not suggest that any attempt be made to use either sub-specific type of *A. pilosellae* as a candidate agent for *P. aurantiaca*. Based on available data, this species is a very infrequent host of *A. pilosellae*. The association is known from only one galled plant and we found no further mentions in the literature. We did not find *P. aurantiaca* to be galled anywhere in our survey area, despite being the most abundant *Pilosella* species present at multiple sites where *A. pilosellae* was also present.

**Limitations and suggestions for future genetic study of *Aulacidea pilosellae***

We interpret patterns of cryptic host-associated differentiation of *A. pilosellae* using all data available to us; however, our results indicate there may be further genetic differentiation present in *A. pilosellae* not captured by our study. While we attempted to conduct a broad survey encompassing much of the geographic range of *A. pilosellae*, we were constrained by time and resources. Previous work pointed us to central Europe, where we knew many species of *Pilosella* invasive in North America are common, as is *A. pilosellae*. However *A. pilosellae* is also known from other European countries such as Spain (Nieves-Aldrey et al. 2005), the United Kingdom (Eady and Quinlan 1964), Israel (Argaman 1988) and Hungary (Sárospataki 1999), but is only described from *P. officinarum* in these countries. Whether *A. pilosellae* also uses other species of *Pilosella* in these other parts of its distribution is not documented. In light of our findings it would be very interesting to obtain more specimens of *A. pilosellae* from other parts of its distribution for DNA sequencing. At the time of writing, we have obtained specimens of *A. pilosellae* ex. *P. officinarum* from the United Kingdom, which will be added to our analyses when sequences are available.

Our interpretations of *A. pilosellae* collected from both *P. floribunda* and *P. piloselloides* are limited, as we only found one plant of each galled by *A. pilosellae*. It would also be interesting to obtain more *A. pilosellae* from these species to determine if the alignment patterns we observed are characteristic of *A. pilosellae* collected from these species. While it may seem

contradictory if we consider these as host species while we do not consider *P. aurantiaca* as a host species, the difference is that there are other records corroborating the use of these species by *A. pilosellae*.

While we found no sequence variation at CO1 between specimens collected at the same site at the majority of sites, at site C16 we observed considerable variation in the CO1 region between specimens, and additional variation in the other two gene regions. Interestingly, the patterns of divergence between these specimens were not consistent between gene regions – e.g. in the CO1 region we found L2a to be highly diverged from L2b and L2c, whereas in the ITS region we found L2a and L2b to share the single transition present in either sequence, while the ITS sequence for L2c was identical to a number of specimens from other sites. The level of sequence divergence observed at this site was not observed at any other site, and in consideration of the lack of any additional evidence, we suppose that this may be an indication that higher levels of sequence divergence may exist in a fraction of populations.

We also suggest that further collectors preserve samples of the host plant for molecular analysis. While we attempted to do this, samples were not consistently collected and stored in a way that would allow for this purpose. Although DNA barcodes are not currently available for *Pilosella* spp., there are sequences available in GenBank (e.g. Burgess et al. *unpublished data*) which may serve this purpose, as well as plastid DNA sequences for some species (Gaskin and Wilson 2007). We are currently collaborating on a study which will assess if DNA barcodes can be catalogued for the invasive *Pilosella* in North America.

## *WOLBACHIA* INFECTION

### Implications of host-associated *Wolbachia* infection

We hypothesized that the differences in reproductive mode between the parthenogenetic *A. pilosellae* ex. *P. officinarum* from the Southern Range and the sexual *A. pilosellae* ex. *Pilosella* spp. in the Northern Range may be due to infection with a bacterial endosymbiont, such as *Wolbachia*. Our detection of *Wolbachia* in both the *A. pilosellae* ex. *P. officinarum* from Southern and Northern Ranges supports this hypothesis, however, we caution that despite there being empirical evidence of such patterns of *Wolbachia* induced parthenogenesis in the literature

(Plantard et al. 1998, Plantard et al. 1999), further work should be conducted to determine if *Wolbachia* infection is in fact inducing parthenogenesis in *Aulacidea pilosellae*.

Some authors (e.g. Hurst and Jiggins 2005) reason that the use of mtDNA in phylogenetic studies where *Wolbachia* is present may yield unreliable results, given the potential for *Wolbachia* to drive non-neutral selection of mtDNA in their hosts. In our study, we saw an increase in mtDNA diversity over nuclear DNA diversity, thus it is plausible that this could in part be an artifact of *Wolbachia* infection separating the mtDNA lineages of *A. pilosellae*. However, in light of the biological differences (i.e. the ecological and physiological host ranges) between the *A. pilosellae* ex. *P. officinarum* in lineage 1 infected with *Wolbachia* and the *A. pilosellae* ex. *Pilosella* spp. in lineage 3, we do not believe the divergence in the mtDNA lineages to be merely an 'artifact' of *Wolbachia* infection. While we do not know the driving force behind the sequence divergence in the CO1 region, we believe that our integrative approach provides sufficient evidence that the divergence observed in the CO1 region is associated with biologically relevant differences among the lineages. Thus, whether the divergence of the CO1 region was mediated by *Wolbachia* infection of not, we find sufficient evidence to reason that there are true biological differences between the *A. pilosellae* in lineage 1 and 3, and that while *Wolbachia* infection may be associated with, or even have played a causative role in such divergence, this divergence has biological relevance.

**Limitations and suggestions regarding *Wolbachia*, host use and genetic divergence**

The role of *Wolbachia* in the host-associated genetic divergence of *A. pilosellae* emerges as perhaps one of the most promising avenues for further research coming from this study. While we screened *A. pilosellae* collected from each host-plant species per site per region that were sequenced, we only detected *Wolbachia* in the *A. pilosellae* from *P. officinarum*. Whether *Wolbachia* has played any role in the divergence of the two sub-specific types of *A. pilosellae* cannot be interpreted from our data, although the correlation between *Wolbachia*, sequence divergence and host use of *A. pilosellae* suggests there may be interesting relationships to explore in this group.

Deducing the role of *Wolbachia* in the divergence and host species associations of *A. pilosellae* will likely not be a simple endeavour. We suggest a starting point may be to first quantify rates of *Wolbachia* infection in populations of *A. pilosellae*, following a similar approach to Plantard

et al. (1998). While *Wolbachia* infection rates are usually high (>90%) within those Cynipidae populations affected (Plantard et al. 1998, 1999), rates as low as 70% have been reported. Thus, we believe it may be important to sample more individuals per collection site to better assess *Wolbachia* infection rates and be more confident that the lack of detection represents true absence in populations. In our study, we only tested one individual per collection site in most cases. Further, we suggest curing *Wolbachia* infection with anti-biotics, as has been done in many studies (Plantard et al. 1998, Zchori-Fein et al. 2000) to determine how infection loss can alter both reproductive mode and behaviour. It would be most interesting to determine if the loss of *Wolbachia* infection could alter the species of *Pilosella* accepted as hosts of *A. pilosellae*, or if inducing *Wolbachia* infection would also have an effect. Zchori-Fein et al. (2001) cured *Wolbachia* infection in the parasitoid wasp *Encarsia pergandiella*, and observed that cured females accepted one host species with the same frequency as the control (infected) group, but accepted another host species significantly less than the control group. Thus, there is some empirical evidence that *Wolbachia* infection (or at least loss of infection) can alter host acceptance in the Hymenoptera.

## POTENTIAL UTILITY OF DNA BARCODING TO BIOLOGICAL CONTROL

The Barcode of Life Data System (BOLD, Ratnasingham and Hebert 2007), or DNA barcoding, has emerged as one of the most highly funded and controversial biological investigations ever attempted (Cameron et al. 2006). Proponents argue that one utility of DNA barcoding lies in its ability to rapidly assess biodiversity (Smith and Fisher 2009, Smith et al. 2009); much more quickly than traditional morphology-based taxonomy. While we are aware of cases where DNA barcodes were used to identify new exotic and/or invasive species (deWaard et al. 2009), we are not aware of any such cases where that exotic species was in fact a candidate biological control agent for an existing invasive species. While we have far from definitive evidence that the CO1 sequence we obtained from GenBank (accession no. JN288739) is in fact *Aulacidea pilosellae*, we would not have begun to expect that this species, known only from European records, could be present in North America without this publicly available record. As we previously suggested, we are interested in obtaining sequence data from a nuclear gene region, and if it also aligns with our *A. pilosellae* sequences from Europe, we believe the next steps should be sending the specimen to the appropriate taxonomist and beginning searches on *Pilosella* species near the collection location.

If this specimen does prove to be *Aulacidea pilosellae*, it would be the first North American record of the species, and potentially expedite the process for approving this candidate agent for release in North America (De Clerck-Floate, *pers. comm.*). Regardless of the outcome of this particular case, there is clear potential significance to biological control. By instituting DNA sequencing of candidate agents and 'blasting' them against available sequences in databases such as GenBank, the number of years required to petition candidate agents could potentially be reduced if it were found that the species already was present (but not documented in the literature) in the country to which is was going to be released. We believe this provides an additional potential benefit of mass DNA sequencing projects, and that we may be the first to make such a suggestion.

## CONCLUSION

This thesis assessed the potential for host preferences, the ecological host range, and patterns of cryptic genetic diversity in *Aulacidea pilosellae* Kieffer (Hymenoptera: Cynipidae). The overarching goal of our study was to test hypotheses related to the patterns of host species use of this candidate weed biological control agent, in order to inform and benefit the biological control programme for *Pilosella* hawkweeds invasive in North America. *Aulacidea pilosellae* has received little scientific study, as such the contributions of this thesis significantly advance our knowledge of this species, yet much more work is required to better understand the basic biology of this species, and mechanisms of host selection. We were able to confirm that *A. pilosellae* uses multiple species of *Pilosella* as hosts, several of which are invasive in North America. We further found compelling evidence to conclude there are at least two sub-specific types of *A. pilosellae* that have divergent mtDNA sequences and differ in host species use, in infection with the bacterial endosymbiont *Wolbachia*, and in reproductive mode, yet the extent to which these differences are related and potentially cause one another remains to be examined.

Based on the results of this study and the body of work conducted by Grosskopf (2002, 2003, 2004), we suggest that further efforts in the *Pilosella* hawkweed biological control programme regarding *A. pilosellae* prioritize testing *A. pilosellae* ex. *Pilosella* spp. from Region C of our study area as a candidate agent for *P. caespitosa* and *P. glomerata*, as these species are widespread and troublesome in the introduced range, and common hosts of *A. pilosellae* in the Northern Range. We also suggest that if an additional biocontrol agent is desired for *P.*

*officinarum* (one has been released), that *A. pilosellae* ex. *P. officinarum* from the Southern Range be used, and only used as a candidate agent for this species. We believe there may be potential for *A. pilosellae* ex. *Pilosella* spp. from the Northern Range to provide control of members of the *P. piloselloides* aggregation, including *P. piloselloides* subsp. *praealta*, as well as *P. floribunda*, and less likely (due to no conformation in our study or Grosskopf's) *P. flagellaris*. We do not believe *A. pilosellae* should be considered as a candidate agent for *P. aurantiaca*, due to the lack of use of this species despite its sympatric occurrence with *A. pilosellae*.

Our study is among the first to integrate systematic evaluations of host species use across a broad geographic distribution of a candidate weed biocontrol agent in its native range with molecular phylogenetics, prior to release. We believe this approach has demonstrated a number of advantages that will expedite and inform the *A. pilosellae-Pilosella* biological control programme, and that a similar approach should become part of the standard pre-release assessment protocols of candidate agents.

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