Title
Population Genetics in Biological Control: Cryptic Species, Host-associations, and the Geographic Mosaic of Coevolution

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Publication Date
2015

Peer reviewed|Thesis/dissertation

Population Genetics in Biological Control: Cryptic Species, Host-associations, and the Geographic Mosaic of Coevolution

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Environmental Science Policy and Management

in the

Graduate Division

of the

University of California, Berkeley

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Spring 2015

ABSTRACT

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In this dissertation I expand upon our knowledge in regards to the utility of population genetic approaches to be used for the study of the evolution of introduced biological control agents and their target pests. If biological control methods are to provide sustainable pest management services then more long-term studies will be necessary, and these studies should also include the use of population genetic approaches. For existing biological control programs, post-release population genetic studies could be initiated using museum voucher specimens for baseline data. In Chapter 2, I explored what factors influence our ability to extract usable genomic material from dried museum specimens, and whether we could use non-destructive techniques for parasitic hymenoptera. I found that the age of the specimen was the most important determinant for the amplification of PCR products, with nuclear loci having a higher probability of amplification from older specimens than mitochondrial loci. With these sequence results I was able to differentiate voucher specimens of different strains of the biological control agent *Trioxys pallidus* and I was able to confirm the identification of an unknown parasitoid reared from the invasive light brown apple moth.

For population genetic surveys to be conducted more frequently in biological control programs, some of the barriers to developing molecular markers that are variable enough for these types of surveys need to be overcome. One barrier is the time required to develop polymorphic microsatellite markers. Therefore, in Chapter 3, I developed a novel bioinformatics pipeline that searches through next-generation sequence (NGS) data and uses the raw sequencing reads to identify polymorphic loci. Using this approach I was able to rapidly develop microsatellite markers for two of my study species (*T. pallidus* and *Chromaphis juglandicola*). For both species more than 60% of the target markers amplified and were found to be polymorphic, compared to previous approaches where the success rates were much lower (published studies often show rates between 1 and 20%).

I then examined evolutionary factors that may affect the sustainability of two classical biological control programs; 1) the biological control of walnut aphids, and 2) the biological control of invasive knotweeds. The walnut aphid biological control program is a textbook successful biological control program, but has shown recent evidence of localized breakdowns,

whereas the biological control program for invasive knotweeds is currently under review in the United States and Canada. In Chapter 4, I explored whether hybridization between introduced "strains" of *T. pallidus* is responsible for recent breakdowns in this control program. In that study I found low levels of hybridization – thus it is unlikely hybridization is playing an important role in these breakdowns – as well as evidence that two of the strains may actually be cryptic species; one being a specialist and another being a generalist. In Chapter 5, I explored whether the geographic mosaic theory of coevolution might help explain these localized breakdowns. In that chapter I found evidence for a geographic mosaic in the walnut aphid biological control program, and commented on how components of the geographic mosaic theory of coevolution can help us predict what systems we might expect localized breakdowns to occur in. In Chapter 6, I explored whether endosymbionts might play a role in shaping the host-associations of two strains of the candidate biological control agent for invasive knotweeds. I found that while strains of the psyllid *Aphalara itadori* showed no barriers to hybridization of their nuclear genomes, there were curious patterns of horizontal transmission of their primary endosymbiont. I also found that one haplotype of the secondary endosymbiont *Sodalis* sp. dramatically changed in frequency during the hybrid crosses reared on giant knotweed. When compared with previous studies of this species, the results I observe suggest that endosymbionts may play an important role in the differing fitness levels of these two strains.

In conclusion, population genetic approaches provide valuable tools for the study of post-release dynamics in biological control settings. While biological control programs promise to be useful study systems for evolutionary interactions, post-release studies will allow for that promise to come to fruition. In my future research endeavors I would like to continue to monitor the effects of hybridization and the frequency of geographic mosaics of coevolution in biological control settings. In addition, I would like to conduct post-release population genetic studies of both previous successful introductions and programs that resulted in failures. I believe these post-release studies will allow us to better determine what evolutionary factors affect the sustainability of biological control services and will allow for better management practices.

Dedication

For Michelle

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ACKNOWLEDGEMENTS

First, I cannot thank my guiding professor Dr. Nicholas J Mills enough for his support throughout my dissertation research. He met every stumbling block – and I know I created more than were necessary – with a smile, a laugh, a smile and a laugh, or a "well, that's not very good!" I'll miss coming into the office and knocking on the door, as his heart-felt good mornings provided the perfect start to each workday. When I was looking for graduate programs, a colleague stressed how important having a good relationship with your guiding professor would be. When I heard Nick's laugh during our first phone conversation I knew that he was the right person to work with – and he was. I'm sure he'll appreciate a little more free time now that he no longer has to turn my half-broken writing (and thoughts) into sentences, but I'll forever be grateful for his help, his enthusiasm, and for the opportunities he's provided.

I cannot imagine how any of this would have been possible without my family and friends. For Michelle; my partner, my biggest supporter, my vocalist critic, my beautiful wife, and the world's best field assistant – though the fact that you collect twice as many insects at each location is a little embarrassing for me as the "entomologist." You opened my eyes to the beauty of forests and mountains, to vernal pools and to oak savannahs, and everyday that we spend together I'm struck by how luck I am. From our night walks in the hills, to camping by mountain-rivers, thank you for picking me to share this wonderful journey with. For my parents; thank you for always being a phone call away, for your faith in me, for instilling in me a love of what's "good and green" in this world, for teaching me to work with my hands and to think with my heart, for Ski Mother Earth and for Earth Day cleanups, I'm very a lucky son. For Ron and Linda and the little doggies; thank you for welcoming me into your family. You're without a doubt the best in-laws that anyone has ever had. For Myrrha and Christi; my loving sisters, thank you being there at all hours of the day while I walked to campus, I can't wait to spend more time with you. I am forever grateful for being your brother and for your creativity, support, and guidance. For Adam, Brett, and Joe; my fantastic brother-in-laws, hopefully now that I'll finally be graduated I can start to give back in kind for all of your generosity and love. For Margot, Isla, and Aria; my beautiful nieces. I'm so happy to be your uncle and I hope that years of entomology themed presents will do the trick in bring you over to the bug world. For Monique; merci pour tout votre amore. I'm sure you were starting to wonder if your oldest grandchild would ever graduate, thank you for never loosing hope! For the extended Andersen and Stillger families, thank you! For Dorothy and the Spruce St neighbors; you've made Berkeley our home, thank you for welcoming us into your lives and your families!

This work also could not have been possible without the generous support of the Environmental Protection Agency whose STAR fellowship gave me the opportunity to ask questions and the resources to address them, as well as the van den Bosch Memorial Scholarship that supported and encouraged much of this work. I'd also like to thank the University of California Cooperative Extension Specialists who assisted me in my many poorly prepared field collecting endeavors, the individual walnut and filbert growers who allowed me access to their orchards, and one particularly colorful French grower who, though my French is a little rough, I think told me, "there are no aphids here, if there are aphids here you brought them with you from America." The orchard was hammered with aphids, and I know I didn't bring them with me. His statement fills me with joy every time I think about it.

Finally, thank you to my dissertation committee members. You made working on this project a pleasure, and I hope that this is the start of many more collaborations to come.

CHAPTER 1: INTRODUCTION

Background

Classical biological control (hereafter referred to simply as biological control), the reduction in abundance of invasive pestiferous insects and/or weeds through the use of introduced natural enemies (Van Driesche *et al.* 2010), is often considered a sustainable form of pest management (Bianchi *et al.* 2006; Bale *et al.* 2008) due to the fact that it does not rely upon limited natural or synthetic resources. However, too often biological control programs have been initiated and natural enemies introduced without the use of long-term studies to verify their persistence and efficacy as control agents through time (Mills 2000; McCoy & Frank 2010). In many regards this lack of long-term studies is a simple reflection of the fact that biological control is a form of pest control service that is conducted across a landscape, and thus generally not marketable to individual growers (though augmentative biological control programs initiated in greenhouse settings provide fine examples of exceptions [van Lenteren & Woets 1988; Paulitz & Belanger 2001]). As such, these projects are usually funded by governments (e.g. local, regional, and or national governments) or in some instances by crop marketing boards whose needs often necessitate a focus on new and emerging pests rather than on the re-evaluation of previous successes and/or failures. However, research universities may be particularly well situated to provide the ideal location to conduct both post-release and long-term studies of biological control services, and a greater understanding of the factors responsible for both biological control program successes and failures will greatly improve our ability to provide sustainable pest control services.

The necessity for these analyses is all the more vital in the face of increasing levels of biotic invasions (Mack *et al.* 2000; Ricciardi 2007; Kumschick *et al.* 2015). In response, biological control programs – where a non-native and potentially invasive pest is reunited with a natural enemy from its region of origin (Hoddle 2004) – are often used to reduce the ecosystem impacts of the invader. In the nearly 130 years since the successful introduction of the vedalia beetle (*Rodolia cardinalis* [Mulsant][Coleoptera: Coccinellidae]) to California for the biological control of cottony cushion scale (*Icerya purchase* Maskell [Hemiptera: Coccoidea]), and with it the beginning of modern biological control (Doutt 1964; Caltagirone & Doutt 1989; Sawyer 2002), the field has been greatly improved. There has been greater emphasis on the use of scientific methods and principles for selecting target pests and candidate natural enemies and conducting risk assessment, particularly pre-release host range testing (e.g. Messing 2001; Sheppard *et al.* 2005). However, due to a consistent lack of post-release and long-term studies, our understanding of how non-native pests and their introduced natural enemies evolve in their novel habitats and what effect those evolutionary changes have on the efficacy of their associated biological control programs is severely limited.

One commonly used practice that may inadvertently, and unexpectedly, influence the evolutionary trajectories and the efficacy of introduced natural enemies has been the collection and importation of natural enemies from multiple geographic locations, hosts, and/or climatic regions. These different source populations are often referred to as strains (Hopper *et al.* 1993; Clarke & Walter 1995) and justifications for releasing a diverse assemblage of genetic lineages

of the natural enemy included; 1) that if one strain was more compatible with the novel habitat or more adapted to the target host the probability of establishment would be increased, and 2) that through interbreeding, the established population would be less likely to suffer from genetic bottlenecks.

However, recent molecular work with a number of insect taxa have highlighted the common occurrence of cryptic species complexes in what were previously thought to be a single species (Campbell *et al.* 1993; Hebert *et al.* 2004; Janzen *et al.* 2005; Smith *et al.* 2006). In addition, cryptic species are known to have played a nefarious role in either preventing or delaying the implementation of biological control programs (DeBach 1960; Cox & Williams 1981; Room 1990). Therefore, it is likely then that many of the "strains" that were used in classical biological control programs were actually cryptic species complexes. If we are to better understand the factors that influence the establishment of different strains, it is first important to have an accurate delimitation of species boundaries. In addition, even though there may have been a desired goal of genetic mixing between strains, it is unclear what effects hybridization may have on the efficacy of biological control agents. Hybridization, while once considered rare among animal taxa (Mayr 1942), is increasingly being shown to be a common occurrence and an important driver of evolution (Mallet 2005) – particularly in the Insecta (Schwenk *et al.* 2008) – and rates of hybridization have been increasing due to anthropogenic influences (Allendorf *et al.* 2001). Interestingly, both increased fitness (i.e. hybrid vigor), and reduced fitness (i.e. hybrid breakdown) are possible following hybridization, and hybridization has been shown to be a concern in conservation settings where the ensuing hybrid swarms (with their reduced fitness) complicate and delay the preservation of target populations (Allendorf *et al.* 2001). It is unclear what the effects of hybridization have been on the success of biological control introductions and the sustainability of biological control services. However, a previous meta-analysis of biological control programs showed that those which used multiple strains were twice as likely to fail as those that used only a single source population (Clarke & Walter 1995). Being that it is likely that hybridization occurred between the strains (either during collection and shipping, or after release), hybridization may have played a role in these past failures.

For my dissertation I chose to study two classical biological control systems. For the first part of my dissertation I conducted post-release genetic surveys of both hosts and parasitoids associated with the biological control program for the walnut aphid, *Chromaphis juglandicola* (Kaltenbach) (Hemiptera: Aphididae). Walnut aphids were once the principle pest in walnut orchards in California (a crop that occupies >113,000 ha and is valued at ~1.8 billion USD in California alone; USDA 2014), where they are active from March through early December (Sluss 1967) and negatively effect tree health as well as the quality and size of the nuts produced (Michelbacher & Ortega 1958). Because of economic concerns, the parasitoid wasp *Trioxys pallidus* (Haliday) (Hymenoptera: Braconidae) was identified in Europe and introduced to the walnut growing regions of California (Schlinger *et al.* 1960). However, while this introduction resulted in establishment in the southern and coastal regions of California, *T. pallidus* failed to establish in the primary walnut growing region of the Central Valley (van den Bosch *et al.* 1962) leading to a second, and ultimately successful, introduction from Iran (van den Bosch *et al.* 1979). There have, however, been recent reports of localized breakdowns in this biological control system, and the reasons for these breakdowns are unkown (Hougardy & Mills 2008).

For the walnut aphid biological control system I had the following research objectives. First, I wanted to see whether voucher specimens located in the Essig Museum of Entomology can be used in conjunction with non-destructive DNA extraction techniques to examine the level of genetic variability present in the founding populations while preserving the vouchered specimens for future morphological analyses. This study was conducted to determine the feasibility of the approach and the factors (e.g., age or size) that might influence the ability to obtain DNA from museum specimens (Chapter 2). Second, I wanted to examine the population structure and hybridization rates between the different strains of *T. pallidus* in their native and introduced ranges. To conduct this survey I developed a novel bioinformatic pipeline for identifying polymorphic microsatellite loci from Next-Generation Sequencing datasets to develop microsatellite loci for both *T. pallidus* and *C. juglandicola* (Chapter 3). I then used this approach to explore whether the two strains of *T. pallidus* introduced to California, and a third strain introduced to Oregon for the biological control of filbert aphids, *Myzocallis coryli* (Goeze)(Hemiptera: Aphididae)(Messing & AliNiazee 1988, 1989), represent cryptic species and to estimate hybridization rates between these strains in their native and introduced ranges (Chapter 4). Third, I wanted to explore whether a geographic mosaic of coevolution (Thompson 1999, 2005) may be occuring in the walnut aphid biological control program (Chapter 5), and whether oscilattions between coevolutionary hotspots and cold spots might have implications for the sustainability of biological control services.

For the second biological control system, I chose the proposed program for invasive knotweeds, *Fallopia* spp. (Caryophyllales: Polygonaceae), by the psyllid *Aphalara itadori* Shinji (Hemiptera: Psyllidae). Knotweeds were first introduced to North America in the mid 1800's (Beerling *et al*. 1994), have subsequently become an invasive weed along riverbanks, roadways, wetlands, and other disturbed areas (Maerz *et al.* 2005; Siemens & Blossey 2007), and are responsible for reductions in native plant and animal species diversity (Gerber *et al.* 2008; Murrell *et al.* 2011). Similarly to the walnut aphid biological control program, multiple strains of *A. itadori* from Japan have been proposed for introduction. Therefore I wanted to develop molecular markers to 1) identify whether these strains represent cryptic species, 2) to create the necessary resources to conduct post-release analyses of hybridization rates between the strains if *A. itadori* is approved for release, and 3) to explore whether endosymbionts may play an important role in creating reproductive barriers between the strains (Chapter 6).

Finally, in Chapter 7, I conclude by commenting on the need for post-release studies of biological control programs that have resulted in both success and failure. I also comment on the utility of population genetic approaches for studying evolution in biological control systems, and what the results from this study may indicate for sustainable pest management strategies.

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CHAPTER 2: DNA EXTRACTION FROM MUSEUM SPECIMENS OF PARASITIC HYMENOPTERA

Abstract

At the same time that molecular researchers are improving techniques to extract DNA from museum specimens, this increased demand for access to museum specimens has created tension between the need to preserve specimens for maintaining collections and morphological research and the desire to conduct molecular analyses. To address these concerns, we examined the suitability of non-invasive DNA extraction techniques on three species of parasitic Hymenoptera (Braconidae), and test the effects of body size (parasitoid species), age (time since collection), and DNA concentration from each extract on the probability of amplifying meaningful fragments of two commonly used genetic loci. We found that age was a significant factor for determining the probability of success for sequencing both 28S and COI fragments. While the size of the braconid parasitoids significantly affected the total amount of extracted DNA, neither size nor DNA concentration were significant factors for the amplification of either gene region. We also tested several primer combinations of various lengths, but were unable to amplify fragments longer than ~150 base pairs. These short fragments of 28S and COI were however sufficient for species identification, and for the discovery of within species genetic variation.

Introduction

Methods for extracting and analyzing DNA sequence data from specimens not immediately preserved for DNA extraction are improving at a rapid rate, as highlighted by the recent sequencing of the Neanderthal genome (Green *et al.* 2011). Among these methods, several techniques exist which allow DNA to be extracted from a specimen without conferring visible damage (Gilbert *et al.* 2007; Rohland & Hofreiter 2007; Rowley *et al*. 2007). These "non-invasive" techniques are of particular interest to natural history museums as they have the potential to contribute to the value of collections, with little to no cost to the museum with regard to the number and quality of specimens held. Insects are a group where these techniques have received increasing attention, and non-invasive techniques have been used for a variety of orders, including Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera, as well as several non-insect arthropods belonging to the Acarina and Aranea (Gilbert *et al*. 2007; Rowley *et al.* 2007; Nagy *et al.* 2010; Bluemel *et al.* 2011; Lis *et al.* 2011; Tagliavia *et al.* 2011). Recent attempts have been able to amplify, through polymerase chain reaction (PCR), useable fragments of mitochondrial DNA from insect specimens collected as early as 1820 (Thomsen *et al.* 2009). DNA extracted from museum specimens has been a useful source of information for understanding recent shifts in population structure, especially with regard to population declines in native pollinators (Lozier & Cameron 2009; Strange *et al*. 2009), in addition to having been helpful in the context of molecular based identifications (Rowley *et al*. 2007), and the short fragments of DNA extracted from museum specimens have recently been used in Next-Generation Sequencing applications (Shokralla *et al.* 2011).

Unfortunately, due in part to the increased demand by researchers for access to museum specimens, tensions exist between the need to preserve specimens for morphological research

and the desire to conduct molecular analyses (Mandrioli 2008). Part of this tension is a result of a general lack of knowledge on behalf of both researchers and museum curators as to the likelihood of successfully extracting DNA from dried specimens, the likelihood of generating meaningful sequence data for subsequent analysis, and the post-extraction quality of museum specimens used for non-invasive techniques.

One taxon for which DNA information from museum specimens is highly desirable is the parasitic Hymenoptera, in which cryptic variation is common and correct identification is notoriously difficult - even for trained specialists (Noyes 1994). In addition, parasitic Hymenoptera have been the subject of many phylogenetic and evolutionary studies (Dolphin & Quicke 2001), and are important economically, because of their value in the biological control of insect pests in agricultural, urban, and forest environments (Mills 2000; Van Driesche *et al.* 2008).

In this study we examine the suitability of non-invasive DNA extraction techniques for pinned specimens of three species of parasitic Hymenoptera (Braconidae). We test the effects of body size (parasitoid species), and age (time since collection) on the total amount of DNA extracted, and the effect of these three factors on the probability of amplifying meaningful fragments of two commonly used genetic loci. We then test the utility of these amplified fragments in conjunction with previously published sequences for producing phylogenetic trees, one of the primary methods for species identification, and discovery of within-species genetic variation (Goldstein & DeSalle 2010). Finally, we make recommendations regarding the suitability of non-invasive techniques for molecular analysis of less robust museum specimens.

Methods and Materials

Species Examined

Specimens from three species in the family Braconidae (*Atanycolus longifemoralis* Shenefelt, *Meteorus trachynotus* Viereck, and *Trioxys pallidus* Haliday) were selected from the collection of parasitic Hymenoptera housed in the Essig Museum of Entomology at the University of California, Berkeley. Permission to work with specimens was granted by the Essig Museum, and all specimens were provided on loan. These three species are represented by a large number of specimens collected over a range of years, and by individuals that have been identified by taxonomic specialists. *A. longifemoralis* is a large (2–8 mg dry weight) ectoparasitoid of wood-boring coleopteran larvae, such as *Melanophila drummondi*, found on Douglas-fir in the west United States, and British Columbia (Shenefelt 1943; Scott 1974; Deyrup 1975). We examined 15 specimens of *A. longifemoralis* collected between 1931 and 1981. *M. trachynotus* is a midsize (0.1-0.3 mg dry weight) endoparasitoid of *Choristoneura* budworms in North America (Thireau *et al.* 1990). We examined 6 specimens of *M. trachynotus* collected either in 1914 or 1980. We also examined three unidentified specimens in the genus *Meteorus* collected in 2009. *T. pallidus* is a small (< 0.03 mg dry weight) endoparasitoid that was introduced to California and Oregon for classical biological control programs of walnut (*Chromaphis juglandicola*) and filbert (*Myzocallis coryli)* aphids respectively (van den Bosch *et al.* 1962; van den Bosch *et al.* 1970; Messing & AliNiazee 1989). We examined 12 specimens of *T. pallidus* collected between 1959 and 1993. For all specimens, collection information is provided in Table 2.1.

DNA Extraction Protocol

The general practice for extracting DNA from "ancient" specimens is to use a sterile laboratory – a space where no previous molecular work from the taxon of interest has been performed. However, if DNA extractions are to be routinely performed on insect specimens from museum collections, such as those housed in the Essig Museum, it is unlikely that new sterile laboratories will be available for each extraction event. Therefore, we used procedures we believed would minimize the risk of contamination. In addition to standard laboratory practices, all working spaces and instruments, including pipettes, were cleaned with a 10% bleach solution and allowed to air dry prior to extractions. DNA extraction was performed using the buffers and protocols described by Gilbert *et al.* (2007) except as noted. Different methods were used to remove the specimens from their mounts. For specimens that were pinned directly, we first warmed the extraction buffer and then pipetted the warmed buffer over the pinned insect. After several minutes, gentle downwards pressure was applied using flamed sterilized forceps. If the parasitoid did not immediately release from the pin, the process was repeated. Some specimens of *M. trachynotus,* and all of the specimens of *T. pallidus* were glued to mounting points. For these individuals, warmed extraction buffer was used to loosen the bond between the card and the specimen. If after 30 min the parasitoid was still attached, flame-sterilized scissors were used to cut a small piece of the card with attached specimen from the rest of the mounting point to enable the specimen to be placed into the extraction buffer. For all extractions, the whole specimen was placed in a 1.5 ml eppendorf tube with 500 µl of extraction buffer. For *A. longifemoralis*, to fully submerge the specimens, multiple washes with the extraction buffer were required. Methods then followed Gilbert *et al.* (2007). The extracted DNA was suspended in 100 µl of DEPC nuclease free water (BioExpress), and its genomic content was quantified using a ND-1000 NanoDrop® (NanoDrop Technologies, Inc.), before being stored at -20°C.

Remounting of Specimens

After specimens had been in 95% ethanol for at least 12 h they were removed and placed dorsally on a microscope cover slip. Enough ethanol was then added to cover the specimen, and the wings and legs were manipulated and spread prior to remounting. The ethanol was then allowed to evaporate, while the specimen was adjusted with forceps. Specimens were allowed to air dry for at least 48 h before being weighed on a Mettler-Toledo AT21 Comparator microgram balance (Mettler-Toledo International, Inc.). After measurement, individuals of *A. longifemoralis* were re-pinned. For *Meteorus* spp. and *T. pallidus*, the insects were re-glued to mounting points. Specimens were then catalogued for return to the collections at the Essig Museum of Entomology.

DNA Amplification and Sequencing

The ability to amplify two commonly used DNA fragments, the D2 expansion region of the ribosomal gene 28S, and a fragment of the "barcoding region" of the mitochondrial gene Cytochrome Oxidase I (COI), were evaluated. For the amplification of 28S we used the forward and reverse primers, s3660 (Morse & Normark 2006) and 28Sb (Whiting *et al.* 1997), respectively, and two novel forward and reverse primers, Essig28SF2 5' – TTG TCG GCG TGC ACT TCT C – 3' and Essig28SR2 5' – gag aag tgc acg ccg aca a – 3', respectively. For the amplification of COI we used the forward and reverse primers LCO, and HCO (Folmer *et al.* 1994), respectively, one novel forward primer BracCOIF 5' – CAT GCW TTT RTW ATR ATT TTT TTT ATR GTW ATR CC – 3', and three genus specific reverse primers, AtanyCOIR 5' – CTT AAA ATT AAT AAW ATT AAT GAA GG – 3', MeteorCOIR 5' – TTA WAG ATA

AWG GRG GRT AMA CWG TTC AHC C – 3', and TrioxysCOIR 5' – CAA CCC GTA CCA GCC CCT ACA TTT ATT AAA CCC C – 3'. Novel primers were designed using published sequences from congeners in GenBank as a template, and either using the software PriFi (Fredslund *et al.* 2005) or by eye.

Standard PCR protocols were followed using a BioRad Dyad programmable thermocycler (BioRad Laboratories, Inc.). PCR reactions were carried out using Amplitaq GOLD DNA polymerase and buffers (Life Technologies), with the following conditions; 2.5 μl of 10X PCR Buffer II, 1.5 μM of $MgCl_2$, 0.2 μM of dNTP (Promega Corporation), 0.2 μM of each primer, 0.2 μl of Taq polymerase, 1 μl of DNA template, finally H_20 was added to bring the final reaction volume to 25 μl. For the amplification of 28S, all possible primer combinations were tested for all individuals, with an initial denaturing step at 94°C for 4 min was followed by thirty-five cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. This was followed by a 5 min extension step at 72°C. For the amplification of COI, genus specific reverse primers as well as the universal reverse primer "HCO" were used in combination with either the forward primer "LCO" or "BracCOIF" following the touchdown protocol presented by Hebert *et al.* (2003). For all primer combinations, reactions were held at 17°C, and results visualized on a 1.5% agarose gel. Sequencing of both forward and reverse fragments was performed on an Applied BioSystems 3730xl DNA Analyzer (Life Technologies) at the University of California Berkeley DNA Sequencing Facility. Sequence results were edited using Geneious Pro v. 5.5.4 (Drummond *et al.* 2011), and Nexus files containing both sequence data, parameters for phylogenetic analyses, and tree files for each dataset can be found at TreeBase.org (accession number TB2:S12519).

Statistical Analysis

All statistical analyses were performed using the statistical software package R v. 2.14 (R Development Core Team 2011). Differences in DNA concentration (ng/μl) between extracts from parasitoid species were assessed by analysis of covariance (ANCOVA) in the R package STATS (R Development Core Team 2011) with age (in years since collection) included as a covariate. DNA concentration was log transformed to meet assumptions of normality. Backwards, stepwise model simplification was used to examine the significance of interaction terms and main effects, and after simplification, differences in DNA concentration between parasitoid species were assessed using analysis of variance (ANOVA) with Tukey's Honest-Significance test.

To analyze the probability of amplifying meaningful sequences of the two gene fragments, 28S and COI, we performed logistic regression analyses using generalized linear models (GLM), as part of the R package STATS (R Development Core Team 2011), with a Bernoulli distribution (failure/success to amplify either fragment) and a logit-link function, with parasitoid species, age, and log DNA concentration as factors. Multimodel inference was performed based on Akaiki's Information Criterion corrected for small sample size (AICc) (Burnham *et al.* 2011; Grueber *et al.* 2011; Symonds & Moussalli 2011) using the R package AICcmodavg (Mazerolle 2012). Scores were calculated for all model subsets, though the final set of retained models did not include interaction terms due to the extreme differences observed in parameter estimate standard errors (SE) (Agresti & Finlay 2009). Model weights were used to

estimate the relative importance of each of the factors included in the models, and model averaging to provide averaged estimates and confidence intervals for each factor (Burnham & Anderson 2002). As age was the most important factor in the models, simplified models that included age only were used to estimate the effect of age on the probability of amplifying meaningful fragments of 28S and COI for specimens between 0 and 96 years old. These simplified models do not account for all of the variability determined by our multimodel analysis, but may be a useful first approximation in the selection of specimens prior to DNA extraction.

Phylogenetic Analysis

One of the primary methods of analysis to resolve questions of species identification is the production of phylogenetic trees (Goldstein & DeSalle 2010). To be useful for reconstructing accurate phylogenetic relationships, however, sequence fragments must be sufficiently divergent as to differentiate individuals, whilst not being too divergent that their relationships are clouded by too much "noise." For short fragments, this presents a particular problem, and thus quantitative analyses have been performed seeking to optimize the location, length and variability of DNA sequences (Martin *et al.* 1995). To test the utility of short sequence fragments from the two gene regions, to correctly identify known and unknown specimens, as well as to reconstruct meaningful evolutionary relationships between those individuals, we used Maximum Parsimony (MP) to analyze the fragments produced in this study, with sequence data published in GenBank from either the species in question, and/or from congeners. We analyzed both gene regions separately. Alignments were generated using the sequence alignment program MUSCLE (Edgar 2004). For analysis of the COI fragment, due to the high degree of sequence divergence between the three species, individual datatsets for each species (including congeners) were created, again using MUSCLE. Matrices were visualized in MacClade v. 4.08 (Maddison & Maddison 2005), and for all analyses, datasets were truncated to correspond to the sequence fragment generated from our closest primer combinations (Essig28SF2 and Essig28SR2 for 28S; BracCOIF with either AtanyCOIR, MeteorCOIR, or TrioxysCOIR for COI), and primer regions were then excluded. MP analyses were performed using PAUP* v. 4b10 (Swofford 2003) for each matrix using a heuristic search algorithm with a tree-bisection-reconnection branch-swapping algorithm. For the individual analysis of the 28S dataset, gap positions were coded as a 5th character state. Confidence in tree topology was estimated using 1000 bootstrap replicates.

Results

DNA Extraction and Sequencing

Genomic material was extracted from 15 specimens of *A. longifemoralis,* 9 specimens in the genus *Meteorus, and* 12 specimens of *T. pallidus,* with specimens ranging in age at time of extraction from 1 to 96 years. Results from the ANCOVA analysis showed that the total amount of genomic material (DNA concentration) differed significantly between parasitoid species ($F = 10.19$, $df = 2,30$, $p < 0.001$), while age had no effect on DNA concentration ($F = 1.73$, $df = 1,30$, $p = 0.19$), and there was no interaction between age and DNA concentration ($F = 1.06$, $df = 2, 30$, $p = 0.36$). Post-hoc analyses found that DNA concentration differed significantly between specimens of the largest parasitoid species, *A. longifemoralis,* and the smallest parasitoid species, *T. pallidus* ($p < 0.001$) (Figure 2.1).

Of the examined parameters, based on AIC weights from all models, the model with age alone had the largest effect on the success/failure of amplifying 28S and COI (Table 2.2). The sum of the Akaike weights for each model in which age appeared were 0.99 for 28S and 1.00 for COI, compared to 0.31 for 28S and 0.23 for COI for models including DNA concentration, and 0.33 for 28S and 0.3 for COI for models including parasitoid species. In addition, after model averaging, and based on weighted parameter and unconditional standard error estimates, for both 28S and COI, age was the only supported parameter based on 95% confidence intervals (Table 2.3). The logistic regression models using age as the only predictor variable for the amplification of 28S had an intercept of 2.564 ± 0.945 ($t = 2.714$, $p = 0.01$) and a slope of -0.049 ± 0.02 ($t = -2.463$, $p = 0.019$), and for the amplification of COI, an intercept of 2.561 ± 1.125 ($t = 2.275$, $p = 0.023$) and a slope of -0.081 ± 0.03 ($t = -2.689$, $p = 0.007$), see Figure 2.2.

Phylogenetic Utility

For the analysis of the 28S dataset, four MP trees were reconstructed (Figure 2.3). Sequences from all specimens formed clades with sequences from congeneric species published in GenBank with high bootstrap support (B.P.) for *A. longifemoralis* (100% B.P.) and *T. pallidus* (100% B.P.), and medium support for *Meteorus* (74% B.P.). For the analysis of the COI datasets, 13 MP trees were reconstructed for the *A. longifemoralis* dataset, 10 MP trees for the *Meteorus* spp. dataset, and 2 MP trees for the *T. pallidus* dataset (Figure 2.4). Relationships between *A. longifemoralis* and its closest included congener *A. ulmicola* were unsupported. Our unidentified specimens of *Meteorus* formed a highly supported clade (99% B.P.) with published sequences from *M. ictericus,* and our specimens of *T. pallidus* formed a poorly supported clade (65% B.P.) with two published sequences from *T. pallidus*, as well as two published sequences from unidentified Hymenoptera specimens.

Discussion

DNA Extraction and Sequencing

Recently, DNA extracted from insect specimens from museum collections has been used to illuminate questions regarding the population structure and phylogeny of a variety of insect taxa (Gilber *et al.* 2007; Rohland & Hofreiter 2007; Rowley *et al.* 2007; Lozier & Cameron 2009; Lees *et al.* 2010; Nagy *et al.* 2010; Bluemel *et al.* 2011; Lis *et al.* 2011; Shokralla *et al.* 2011; Tagliavia *et al.* 2011; Ugelvig *et al.* 2011). This study is the first to our knowledge to use these techniques with specimens of parasitic Hymenoptera, and the first that attempts to examine the effects of age, size, and DNA concentration of extracts from museum specimens on the probability of successfully sequencing meaningful fragments from those specimens. In general, we found that age had no effect on the amount of total DNA extracted from a braconid parasitoid specimen, but was a significant factor for determining the probability of success for sequencing both fragments of 28S and COI. While specimen size (represented by parasitoid species) significantly affected the total amount of extracted DNA, neither it nor DNA concentration were found to be significant factors for the amplification and sequencing of meaningful fragments of either locus based on 95% confidence intervals.

Some studies (Strange *et al.* 2009; Thomsen *et al.* 2009; Bluemel *et al.* 2011) have reported being able to amplify fragments of DNA from specimens collected more than 100 years ago. While we were able to successfully amplify and sequence short fragments of both 28S and

COI from museum specimens of parasitic Hymenoptera; the oldest specimen from which we obtained 28S was 71 years (collected in 1940), and the oldest specimen from which we obtained COI was 52 years (collected in 1959). In general, we were more successful at amplifying fragments of 28S than fragments of COI, which could be due to a difference in the number of copies of these loci, or even differential rates in which these gene regions are fragmented after an organism's death, though we did not examine either of these possibilities and can only speculate with regard to their importance. Also, compared to Gilbert *et al.* (2007) whose methods we followed, we had a slightly lower rate of success for amplifying 28S (61% compared to 78%) and a much lower rate of success for amplifying COI (38% compared to 71%). Based on our regression analysis, we found that these success rates also decreased with age, with success decreasing at a faster rate for COI than for 28S. We should note that we did not consider the effects of a specimen's temporal history, and assumed that all the specimens in this study were subject to similar storage conditions (temperature, humidity, etc.) while in the Essig Museum. Research has shown that the temporal history of a specimen can affect the success of amplification of DNA from ancient specimens (Smith *et al.* 2011), and researchers examining specimens from multiple natural history collections should consider the possible effects of storage history on their results.

The size of the fragments amplified in this study are similar to those reported in the majority of studies using insect specimens from museum collections (Lozier & Cameron 2009; Lees *et al.* 2010; Bluemel *et al.* 2011; List *et al.* 2011; Shokralla *et al.* 2011; Ugelvig *et al.* 2011), and in particular to that observed by Rowe *et al.* (2011), who found that the majority of the total DNA extracted from their specimens was comprised of fragments between 150 and 300 base pairs, and by Ugelvig *et al.* (2011) who examined the length of microsatellite alleles amplified from museum specimens and found that as specimens increase in age, the length of amplifiable fragments decreases. Conversely, Tagliavia et al. (2011) report being able to amplify fragments of both mitochondrial and nuclear genes of up ~ 800 base pairs from specimens collected 50 years ago, and particularly for phylogenetic studies, their techniques could be of exceptional utility.

While we found no correlation between specimen age and extractable DNA concentration, and DNA concentration was not a significant factor for fragment amplification and sequencing, we caution that it may play an indirect role in the success of amplification based on the following four concerns; 1) that as a specimen ages, total DNA from the specimen may remain unchanged but become increasingly fragmented, thus rendering it unusable for PCR, 2) that as a specimen ages, total DNA from the specimen itself decreases, but over-all DNA concentrations can remain unchanged as bacteria or fungi growing in or on the specimen increase in abundance, 3) that a Nano-Drop, which cannot distinguish between single and double stranded DNA, is not the correct tool for quantifying DNA fragments from critical specimens and alternative methods which only examine double stranded DNA, or include fragment length may be more appropriate, or 4) that residual phenol from the DNA extraction process can mask the true DNA concentration, and that for older specimens these effects may be more pronounced.

Phylogenetic Utility

The phylogenetic analysis of the 28S gene region produced clades that were well supported (Figure 2.3). Our analysis found no difference between specimens of *T. pallidus* at

28S, and a single base pair difference between specimens of *A. longifemoralis*. There was also a single base pair difference between one specimen of *M. trachynotus*, and the other specimens of *M. trachynotus and Meteorus* sp, as well as published sequences for *M. ictericus* and a published sequence from an unidentified *Meteorus*. The fragment of 28S, while not as variable as the fragment of COI we amplified, appeared to be useful for resolving both higher level taxonomic relationships, as well as species level differences between most of the species of *Meteorus* included in this analysis. 28S may not be sufficient however for differentiating between very closely related species (e.g. members of the same species group) as evident from the lack of differentiation between *M. ictericus* and *M. trachynotus*. The fragment of COI that we amplified was more variable than the fragment of 28S (as expected), but was amplified from fewer individuals, and in general more recent specimens (Table 2.1). Using the results of our phylogenetic analysis, we suspect that our unidentified specimens of *Meteorus* sp. are specimens of *M. ictericus* based on the well-supported clade they formed (100% bootstrap support) with all but one of the published sequence for *M. ictericus* by Stigenberg & Ronquist (2011). The one published sequence of *M. ictericus* which was not a member of the clade (HQ264015) was identical to several sequences from *M. ruficeps* and we expect that this represents a labeling error during the GenBank submission process. We also uncovered multiple haplotypes for *T. pallidus* within the specimens stored in the Essig Museum.

Damage to Specimens

Though we did not quantify damage to specimens, unfortunately visible damage was observed for several of the specimens used in this study. *A. longifemoralis* has a long ovipositor, legs, and large wings, and while great care was taken to minimize damage to these structures, the ovipositor sheaths in particular were quite fragile and frequently became dislocated during the DNA extraction process. In all cases, dislocated limbs and ovipositor sheaths were glued to a mounting point on the same pin as the specimen. The major source of damage to specimens was a slight tearing of the wings that resulted from specimens becoming affixed to the glass cover slip during the 48 hr drying period after DNA extraction. All observed damage was done during specimen handling, and was not caused by the DNA extraction process directly. In general, however, specimens did appear to be lighter in color after DNA extraction, and this was most pronounced in the abdomen, though these differences were not quantified. Thus the specific method for DNA extraction used in this study may not be appropriate for specimens for which shades of color is either a distinguishing character or adds to the value of the specimen.

Conclusions

Of the variables we examined in this study, the age of a museum specimen appears to be the most important in determining the probability of amplifying and sequencing meaningful fragments of DNA from parasitic Hymenoptera. We were able to amplify fragments of 28S from older specimens than was the case for fragments of COI. Since 28S exists at a higher copy number than COI, we suspect that as the copy number of a target DNA fragment decreases, the probability of amplifying it successfully from museum specimens will also decrease. Though the DNA fragments produced in this study were relatively short compared to those commonly used for phylogenetic or species identification applications, they were useful both for determining within species variation and for species level identification. For the reconstruction of deeper phylogenetic relationships it may be possible to create “scaffolds” of many short fragments of a

target gene region in order to produce sequence data of sufficient length and diversity for analysis, to create a concatenated matrix of short fragments from two or more gene regions, or to use alternative extraction techniques which may be more effective than the methods examined here at preserving longer fragments of DNA from museum specimens (Tagliavia *et al.* 2011).

Acknowledgements

I am extremely grateful to Cheryl Barr, Peter Oboyski, and Bob Zuparko at the Essig Museum of Entomology at UC Berkeley. I would also like to acknowledge Pete Croucher and Brian Ort for their technical assistance, and Linda Bürgi, Sara Emery, Tim Engelkes, Lisa Fernandez, Julie Hopper, Kevi Mace-Hill, Michelle Labbé, and Kip Will for their constructive comments. I would also like to thank Nicholas Mills, George Roderick, and Rasmus Nielsen for their comments on an earlier draft of this chapter.

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Table 2.1 – Parasitoid specimens from the Essig Museum collection used for DNA extraction, indicating age (years), weight (mg), extracted DNA concentration (ng/ul) and the number of base pairs amplified for each of two selected genetic loci

ID #	Location	Age	Collection	Weight	DNA	28S	COI
Atanycolus longifemoralis Shenefelt							
J0075	Yosemite, CA	79	5.vi.1931	4.013	141.93		
J0076	Fallen Leaf Lake, CA	70	5.vii.1940	8.145	55.04	140	
J0077	Fallen Leaf Lake, CA	70	5.vii.1940	3.763	44.58	140	
J0078	6 mi east of Chester, CA	56	14.vii.1954	5.273	113.53	140	
J0079	6 mi east of Chester, CA	56	14.vii.1954	5.011	66.08		
J0080	6 mi east of Chester, CA	56	14.vii.1954	6.096	310.10		
J0081	Hobart Mills, CA	48	29.vii.1962	5.873	26.08	140	
J0082	7 mi N of Truckee, CA	48	29.vii.1962	2.584	63.36		
J0083	7 mi N of Truckee, CA	48	29.vii.1962	2.780	115.70		
J0084	2 mi W of Brancomb, CA	34	25-27.v.1976	3.225	84.28	140	
J0085	2 mi W of Brancomb, CA	34	25-27.v.1976	4.236	506.16	140	
J0086	2 mi W of Brancomb, CA	34	25-27.v.1976	2*	74.00	140	103
J0087	Echo Lake, CA	29	24.vi.1981	1.588	70.32	140	103
J0088	Echo Lake, CA	29	24.vi.1981	1.739	113.22	140	103
J0089	Tahoe City, CA	29	30.ix.1981	4.425	28.10	140	103
Meteorus trachynotus Viereck							
J0103	Orono, ME	96	27.vii.1914	0.161	13.70		
J0104	Orono, ME	96	27.vii.1914	0.256	152.82		
J0105	Orono, ME	96	26.vii.1914	0.295	5.90		
J0106	La Jara Canyon, NM	30	5.vii.1980	0.122	37.59	139	
J0107	La Jara Canyon, NM	30	5.vii.1980	0.256	34.21	139	
J0108	La Jara Canyon, NM	30	4.vii.1980	0.258	64.50	139	
Meteorus undet							
J0109	Santa Cruz, CA	1	21.5.2009	0.198	78.95	789	658
J0110	San Francisco, CA	1	3.vi.2009	0.274	51.05	789	658
J0111	San Francisco, CA	1	17.vi.2009	0.220	31.40	789	658
Trioxys pallidus Halliday							
J0090	Rancho Santa Fe, CA	51	18.viii.1959	†	11.10	155	128
J0092	Rancho Santa Fe, CA	51	18.viii.1959	†	9.27		
J0093	U.C. Insectary, Albany, CA	47	2.v.1963	†	1.70	155	128
J0094	U.C. Insectary, Albany, CA	47	2.v.1963	†	8.06	155	128
J0095	U.C. Insectary, Albany, CA	47	2.v.1963	†	5.83		
J0096	Citrus Exp. Station, Riverside, CA	33	1977	†	1.30		
J0097	Citrus Exp. Station, Riverside, CA	33	1977	†	84.41		128
J0098	Citrus Exp. Station, Riverside, CA	33	1977	†	11.80		
J0099	U.C. Insectary, Albany, CA	17	13.vii.1993	0.006	4.80		
J0100	U.C. Insectary, Albany, CA	17	13.vii.1993	0.008	13.72	155	128
J0101	U.C. Insectary, Albany, CA	17	13.vii.1993	0.014	11.00	155	128
J0102	Berkeley, CA	17	4.viii.1993	0.023	593.84	155	128

* Specimen weighed on mg scale. † Specimen could not be removed from mounting pin

Table 2.2 – GLM model summaries for the probability of amplifying meaningful sequences of 28S and COI from three braconid parasitoid species. Model names, descriptions, and AIC summaries for supported models examining factors contributing to the amplification of fragments of 28S and COI. K = the number of fitted parameters in the model, AIC_c = AIC score corrected for small sample sizes, Δ_i = the difference between the AIC_c of the current model and that of the model with the lowest AIC_c score, w_i = Akaike weights indicating the probability of the model being the correct model compared to all other tested models.

Model Description	K	AIC_c	Δ_i	w_i	Log-likelihood
28S ~ Age	2	43.83	0	0.43	-19.73
28S ~ Age, Parasitoid species	4	44.85	1.02	0.26	-17.78
28S ~ Age, log(DNA)	3	45.09	1.26	0.23	-19.17
28S ~ Age, log(DNA), Parasitoid species	5	47.47	3.64	0.07	-17.73
28S ~ log(DNA)	2	51.53	7.7	0.01	-23.58
28S ~ Parasitoid species	3	53.94	10.11	0	-23.59
28S ~ log(DNA), Parasitoid species	4	56.19	12.36	0	-23.45
COI ~ Age	2	38.36	0	0.53	-17
COI ~ Age, Parasitoid species	4	39.98	1.62	0.24	-15.34
COI ~ Age, log(DNA)	3	40.64	2.28	0.17	-16.94
COI ~ Age, log(DNA), Parasitoid species	5	42.57	4.21	0.06	-15.29
COI ~ Parasitoid species	3	51.91	13.55	0	-22.58
COI ~ log(DNA)	2	52.45	14.09	0	-24.04
COI ~ log(DNA), Parasitoid species	4	53.16	14.8	0	-21.94

Table 2.3 – Model averaged estimates and uncertainty for the amplification of 28S and COI. Model-averaged parameter estimates were calculated by averaging parameter estimates over all models in which a specific predictor was included. The new averaged parameter estimates are reported with standard errors (SE), as well as 95% confidence intervals (CI). Those parameters whose 95% CI did not include zero are highlighted in bold. Summaries for the categorical parameter Parasitoid species are reported relative to Parasitoid species (*Atanycolus*).

			95% CI	
Parameter	Averaged parameter estimate	Weighted unconditional SE	Upper	Lower
28S				
Intercept	2.785	1.495	5.699	-0.130
Age	-0.054	0.022	**-0.011**	**-0.097**
log(DNA)	0.198	0.329	0.840	-0.444
Parasitoid species (*Meteorus)*	0.186	1.407	2.929	-2.557
Parasitoid species (*Trioxys)*	-1.740	1.035	0.278	-3.757
COI				
Intercept	2.840	1.470	5.707	-0.026
Age	-0.089	0.034	**-0.022**	**-0.156**
log(DNA)	-0.025	0.344	0.646	-0.695
Parasitoid species (*Meteorus)*	-1.798	1.545	1.214	-4.811
Parasitoid species (*Trioxys)*	0.756	1.097	2.895	-1.383

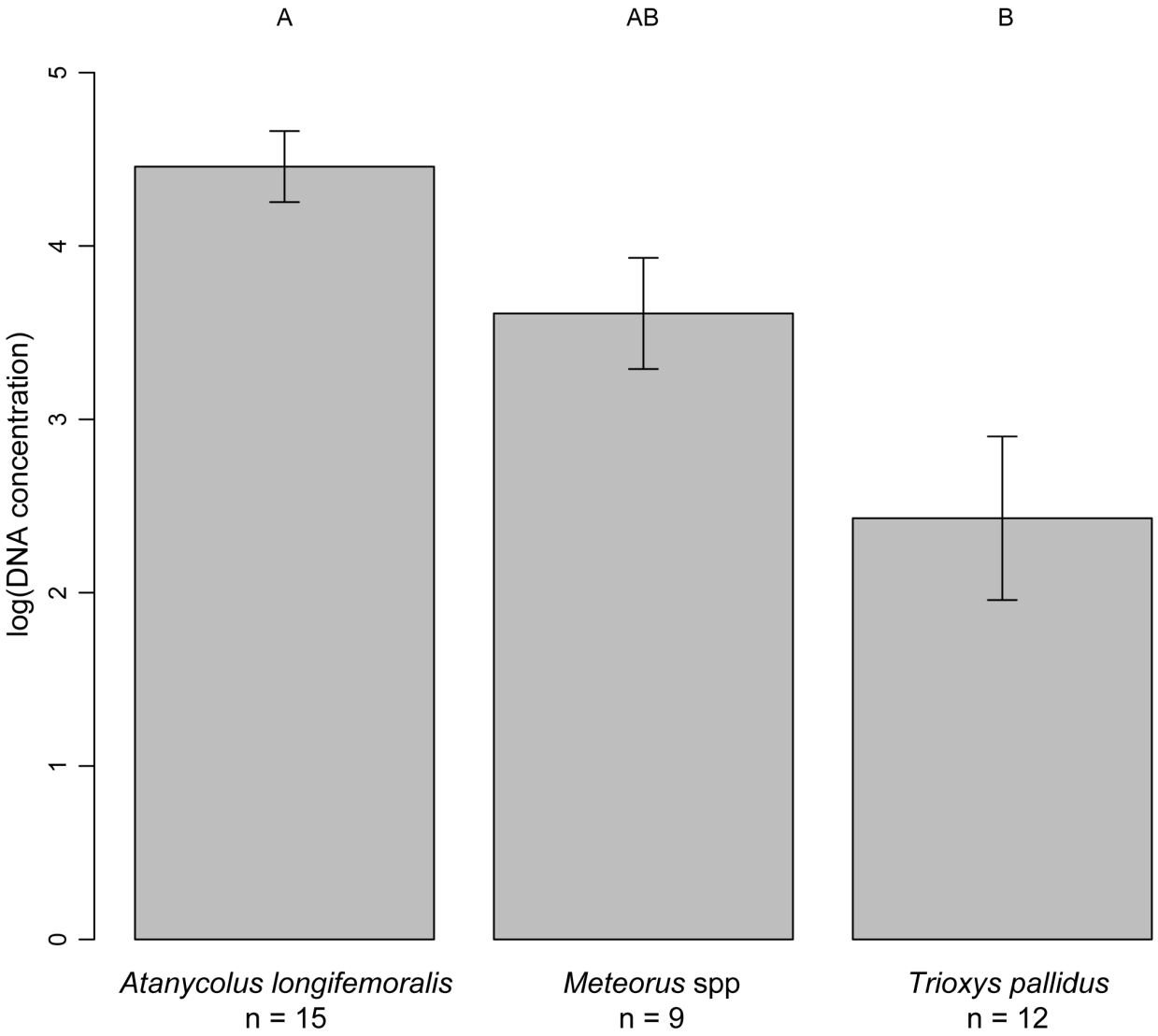

Figure 2.1 – Mean DNA concentrations from three braconid species. Mean (± 1 SE) DNA concentrations (ng/µl) extracted from three braconid species, as measured with a NanoDrop. Statistical differences between the species ($p < 0.05$) are signified by a different letter above each column.

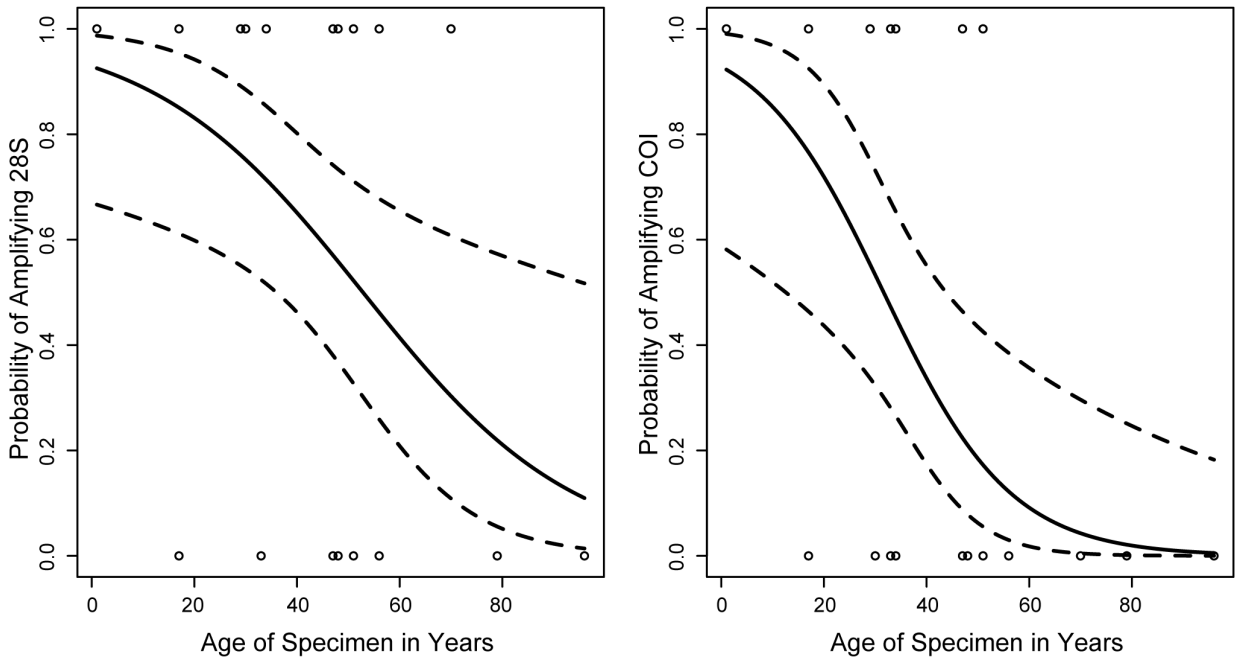

Figure 2.2 – Probability of amplifying fragments of 28S and COI. The probability of successfully amplifying the 28S (left) and COI (right) gene fragments for specimens between 0 and 96 years old were estimated using the results from a logistic regression model with failure/success of amplification of each gene fragment as the response variable and age as the predictor variable. Circles represent the outcome for individual specimens, and the fitted curve from the logistic regression analysis is shown as a solid line, with associated 95% confidence intervals indicated by broken lines. For 28S the intercept equals 2.564 ± 0.945 (t = 2.714, p = 0.01), with a slope of -0.049 ± 0.02 (t = -2.463, p = 0.019), and for COI, the intercept equals 2.561 ± 1.125 (t = 2.275, p = 0.023), with a slope of -0.081 ± 0.03 (t = -2.689, p = 0.007).

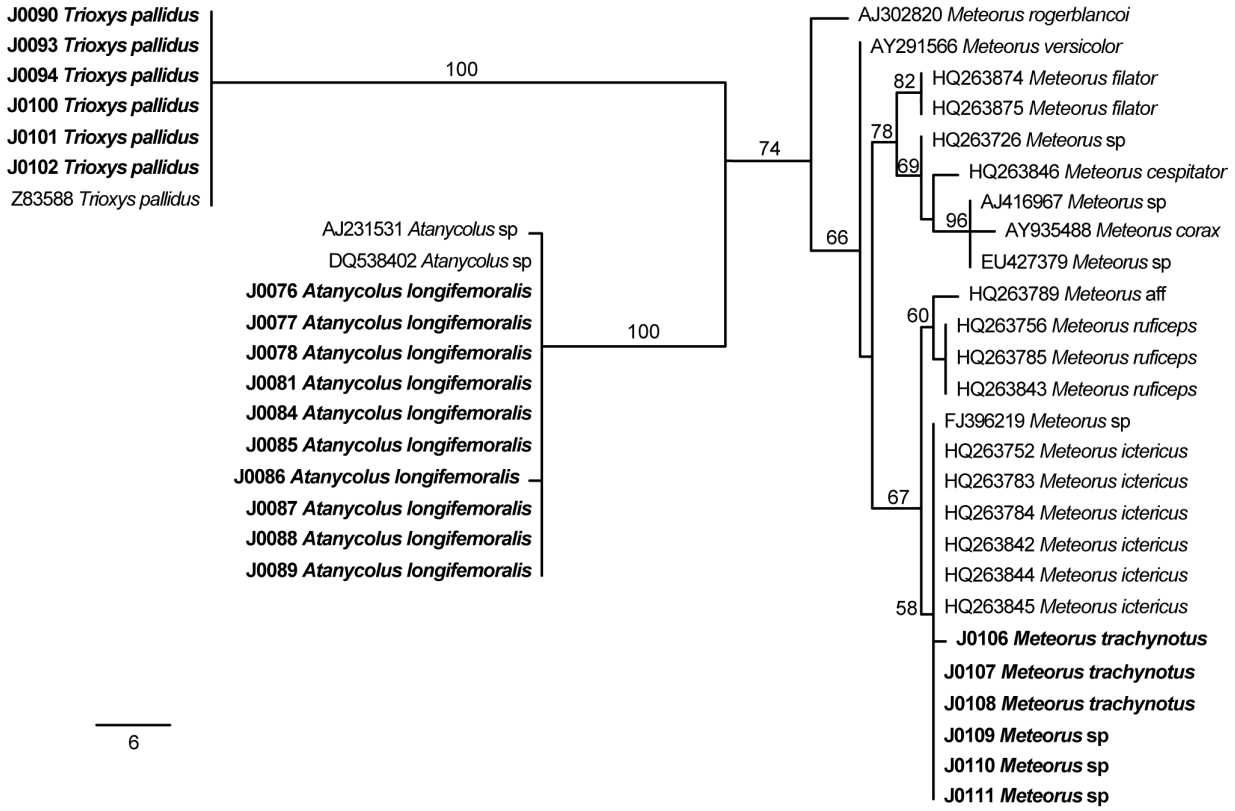

Figure 2.3 – One of eight most parsimonious reconstructions of the 28S dataset. Phylogram showing of one of the most parsimonious trees from the analysis of the 28S dataset. Bootstrap support values are shown either above or next to each supported branch. Sequences generated in this study are in bold. A scale bar indicating branch-lengths is shown in the bottom left.

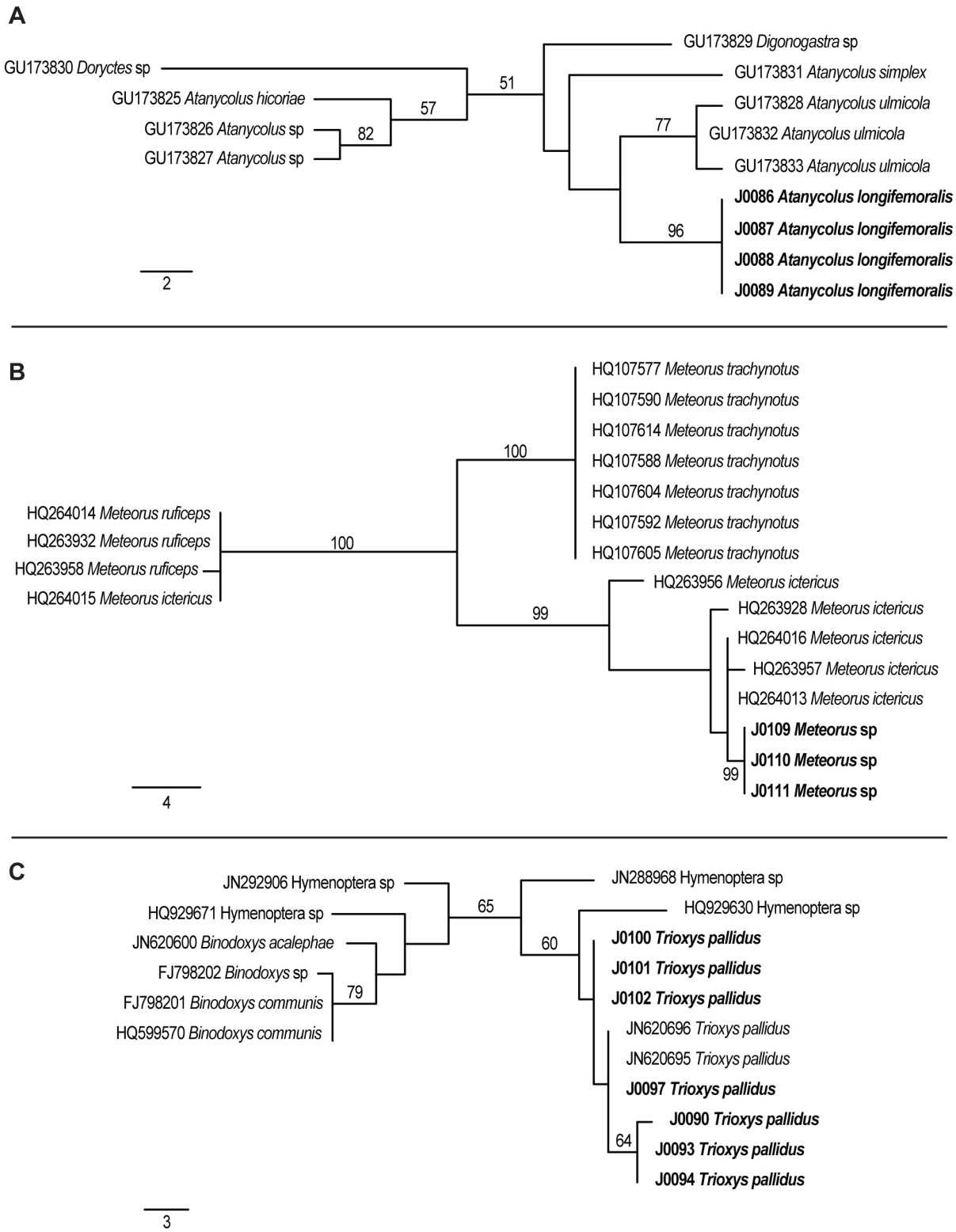

Figure 2.4 – Examples of most parsimonious trees from the analyses of the COI datasets for A) *A. longifemoralis* (1 of 13 MP trees), B) *Meteorus* spp., (1 of 10 MP trees) and C) *T. pallidus* (1 of 2 MP trees). Bootstrap support values are shown either above or next to each supported branch. For each dataset, a scale bar indicating branch-lengths is shown in the bottom left.

CHAPTER 3: iMSAT: A NOVEL APPROACH TO THE DEVELOPMENT OF MICROSATELLITE LOCI USING BARCODED ILLUMINA LIBRARIES

Abstract

Illumina sequencing with its high number of reads and low per base pair cost is an attractive technology for development of molecular resources for non-model organisms. While many software packages have been developed to identify short tandem repeats (STRs) from next-generation sequencing data, these methods do not inform the investigator as to whether or not candidate loci are polymorphic in their target populations. Here we provide a python program iMSAT that uses the polymorphism data obtained from mapping individual Illumina sequence reads onto a reference genome to identify polymorphic STRs. Using this approach we identified 9,119 candidate polymorphic STRs for use with the parasitoid wasp *Trioxys pallidus* and 2,378 candidate polymorphic STRs for use with the aphid *Chromaphis juglandicola*. For both organisms we selected 20 candidate tri-nucleotide STRs for validation. Using fluorescent-labeled oligonucleotide primers we genotyped 91 female *T. pallidus* collected in nine localities and 46 female *C. juglandicola* collected in 4 localities and found 15 of the examined markers to be polymorphic for *T. pallidus* and 12 of the examined markers to be polymorphic for *C. juglandicola*. Our novel approach uses standard Illumina barcoding primers, and a single Illumina HiSeq run to target polymorphic STR fragments to develop and test STR markers. We validate this approach using the parasitoid wasp *T. pallidus* and its aphid host *C. juglandicola*. This approach, which would also be compatible with 454 Sequencing, allowed us to quickly identify markers with known variability and thus presents a significant improvement over existing STR identification software packages.

Introduction

Next-Generation Sequencing (NGS) technologies have recently revolutionized the ease and the rate at which genetic resources can be developed (Mardis 2008; Shendure & Ji 2008; Metzker 2010). This revolution has made it possible to now use the genetic tools that were previously only available for model taxa (Ekblom & Galindo 2011). For example, the development of short tandem repeat (STR or microsatellite) markers in non-model organisms is currently undergoing a complete paradigm shift in regards to the techniques and methods used to isolate potential markers, particularly for insect (Bai *et al.* 2010; Gardner *et al.* 2011; Cerna *et al.* 2012; Keller *et al.* 2012). These new techniques have replaced the laborious steps of DNA cloning with the speed and ease of NGS technologies (López-Uribe *et al.* 2013) allowing researchers to quickly develop candidate markers for their study organisms.

Perhaps as a result of the increased accessibility to NGS technologies for STR marker development, there has also been an increased level of activity in the development of associated software for identifying candidate markers. Many highly cited packages (Edgar & Myers 2005; Li & Wan 2005; Thurston & Field 2005; Jewell *et al.* 2006; Dereeper *et al.* 2007; Kofler *et al.* 2007; Faircloth 2008; Kraemer *et al.* 2009; Castoe *et al.* 2010; Mayer 2010; Castoe *et al.* 2012; Du *et al.* 2013; Miller *et al.* 2013), but see (Lim *et al.* 2013) for a more thorough review. Current software packages work by searching through assembled sequence data for tandem-repeat regions and then apply filters to optimize the list of candidate sites based on user specified criteria. Newer programs directly allow for the use of whole genome data (Du *et al.* 2013) or raw sequence data from paired-end Illumina sequencing (Miller *et al.* 2013). The most recent software program SSR_pipeline represents a particularly important improvement in the identification of STR data by directly using the quality scores from the sequence reads to aid in the identification of STR markers. Yet, two major obstacles remain for the identification of STR

markers for genetic analyses based on NGS sequence results. First, most existing software packages provide an overwhelming number of candidate loci. Second, they do not inform the investigator as to which loci are fixed and which are polymorphic for the populations under study. For example, a recent study that integrated NGS technologies with existing software packages to develop markers for a species of aphid (Jun *et al.* 2012) found that only 0.76% of their 342 candidate markers were suitable for use, though whether this was due to failure to amplify target loci with standard PCR protocols or because amplified loci were not polymorphic is unknown to us.

To improve the rate at which polymorphic STR markers can be identified and developed for use in genetic analyses, we present the use of a novel technique that uses barcoded Illumina sequencing libraries to identify polymorphic STR markers. We test this technique using two insect species from phylogentically distinct orders; the braconid wasp *Trioxys pallidus* and its aphid host *Chromaphis juglandicola.* Both insects occur in walnut orchards in California where *C. juglandicola* is an important invasive pest that was brought under effective biological control by the deliberate introduction of *T. pallidus* from Iran in 1969 (van den Bosch *et al.* 1962, 1970, 1979). We then compare the patterns of STR motifs found for each species to other results published from their respective orders to examine the value of this approach for phylogenetically diverse organisms.

Methods

To identify and test STR markers, we used an NGS approach. Sequencing libraries for *T. pallidus* were created by pooling twenty individuals of *T. pallidus* reared from filbert aphids collected in Bethel, Oregon, United States into a sample labeled "Hazelnut," and twenty individuals of *T. pallidus* reared from walnut aphids collected in Tehran, Iran into a sample labeled "Walnut." DNA was then extracted from each pooled sample using a Qiagen DNeasy Blood & Tissue Kit (Qiagen) with the following modification. To reduce the amount of residual salt in the extract, critical for NGS applications, we performed the AW1 and AW2 washes twice each, followed by an additional spin step to remove any residual AW2 buffer. This was followed by standard elution with the AE buffer. Sequencing libraries for *C. juglandicola* were created by pooling twenty individuals of *C. juglandicola* collected in Upper Lake, California, United States, into a sample labeled "US", and twenty individuals of *C. juglandicola* collected in Parnac, France into a sample labeled "France". DNA was then extracted from each pooled sample using the Qiagen Gentra-PureGene DNA Extraction Kit (Qiagen). Concentrations of nucleic acids for all extracts were then quantified with a ND-1000 NanoDrop® (NanoDrop Technologies, Inc.) and concentrations of double stranded DNA were measured using the Qubit® dsDNA HS Assay kit (Life Technologies Corp.). Sequencing libraries for each *T. pallidus* extract were created using the Nextera™ DNA Sample Prep Kit (Illumina, Inc.) as per Nextera™ instructions, and each library was constructed using a different Illumina barcoding primer. Sequencing libraries for each *C. juglandicola* extra were created using the PrepX™ ILM DNA Library Kit (Wafergen Biosystems Inc.) at The Functional Genomics Laboratory at the University of California Berkeley, and each library was constructed using a different Illumina barcoding primer. Sequencing libraries were examined for fragment length distribution and concentrations using a 2100 Expert Bioanalyzer (Agiliant Technologies), and a KAPA Biosystems Library Quantification Kit (KAPA Biosystems). Each species' libraries were then pooled together, and sequenced independently each using a single run of an Illumina HiSeq2000 (Illumina, Inc.) sequencer at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California Berkeley.

Summary statistics representing the sequence results from the Illumina HiSeq2000 run were calculated using the FASTX-Toolkit (Gordon 2009), and this program was then used to filter low quality reads. Individual Illumina sequencing reads were then assembled into contigs using the *de novo* assembly program Velvet 1.1.06 (Zerbino & Birney 2008) with a kmer length of 65 for *T. pallidus* and 67 for *C. juglandicola*. We then used two commonly used programs MSATCOMMANDER (Faircloth 2008) and Phobos (Mayer 2010) to identify di-, tri-, tetra-, and penta-nucleotide repeat patterns. We ran MSATCOMMANDER and Phobos with their default settings. We then compared the results from these programs to those identified with iMSAT, a novel python program that we developed for this study. This software program is freely available for download at SourceForge (https://sourceforge.net/projects/imsat/). Briefly, the program uses the ".vcf" report file of polymorphic sites generated from mapping NGS sequencing reads to a genome assembly using BWA (Li & Durbin 2009) and SAMtools (Li *et al.* 2009). Both BWA and SAMtools are widely used for the identification and analysis of single nucleotide polymorphism data (McKenna *et al.* 2010; DePristo *et al.* 2011; Nielsen *et al.* 2011).

We have created an interactive command-line interface for the iMSAT program. The program operates as follows (see Figure 3.1 for a graphical representation). The first user prompt asks for the locations of the alignment and ".vcf" files as well as the formatting of the alignment file. iMSAT can process alignment files with both traditional ".fasta" formatted sequence data (i.e. one line beginning with a ">" followed by the sequence name, and a second line with the sequence data) or a tab-delimited format (i.e. one line with both sequence name and sequence data separated by a tab). Our program subsequently filters the "indel" data from the ".vcf" report, and searches for all polymorphic sites that represented di-, tri-, tetra-, and penta-nucleotide STRs that were greater than five repeat units in length. The user is prompted as to whether or not they would like a separate list of polymorphic STR markers that are "fixed" in one of their target populations. The program then produces a ".fasta" formatted file identifying the location of the polymorphic STR in the sequence title and 300 base pairs of both the leading and trailing sequence strands to allow for the production of primers.

For *T. pallidus* we tested the program as if whole genome assembly was being used. To do so, we combined the contig sequences generated by Velvet (Zerbino & Birney 2008) into one continuous DNA sequence strand with the union of two contig sequences being differentiated by the addition of 100 "N" base pairs. The addition of these "N" base pairs ensured that when we could exclude any potential STR markers that would be artificially created when we joined the separate consensus sequences. For *C. juglandicola* we tested the program using the raw output from Velvet (Zerbino & Birney 2008) where all 474,388 contigs were represented in traditional FASTA formatting. For both species we then used the ".vcf" report generated using BWA (Li & Durbin 2009) and SAMtools (Li *et al.* 2009) to target polymorphic STRs.

To validate this approach we further filtered the data to only include those repeat regions that were; a) tri-nucleotide repeats, b) were composed of high-quality reads based on the ".vcf" file, and c) had no "N" base calls within 300 base pairs of the repeat region to allow for primer construction. Though the majority of candidate STRs were di-nucleotide repeats, we selected tri-nucleotide repeats because of the known problems associated with scoring di-nucleotide repeats caused by "stutters" (DeWoody *et al.* 43). For each species we then selected the 20 tri-nucleotide candidate markers with the greatest number of repeat units. Primer pairs for all markers were generated using Primer3 (Rozen & Skaletsky 2000) as implemented in Geneious 5.6.2 (Drummon *et al.* 2012). To ease multiplexing, primers were designed to be at least 20 base pairs in length and to have an optimal annealing temperature of 57°C.

To test the candidate markers, DNA was extracted from 91 female *T. pallidus* reared from three species of aphid (*C. juglandicola* and *Panaphis juglandis* on walnut and *M. coryli* on filbert) from 9 different localities, and from 46 female *C. juglandicola* from 4 different localities (Table 3.1) using the Qiagen DNeasy Blood & Tissue Kit (Qiagen). Non-labeled oligonucelotide primers were used to test and optimize the conditions of each of the 20 candidate regions for each species through standard PCR protocols and the amplified fragments were sequenced at the DNA Sequencing Facility at the University of California Berkeley. For candidate markers that were consistently amplified, fluorescent-labeled primers compatible with the GeneScan™ 600 LIZ size standard (Life Technologies) were used. PCR conditions were then re-optimized for the fluorescent-labeled primers. For both species markers were amplified using one of two PCR protocols signified by their primary annealing temperature (T_a 57 or T_a 50). For T_a 57 an initial denaturation for 5 minutes at 95°C was followed by 35 cycles of 95°C for 60 seconds, 57°C for 90 seconds, 72°C for 60 seconds, followed by a 10 minute extension period at 72°C. For T_a 50, a touchdown protocol was used with the following profile: an initial denaturation for 5 minutes at 95°C, followed by 14 cycles of 95°C for 60 seconds, 57°C for 90 seconds, and 72°C for 60 seconds where the annealing temperature decreased 0.5°C every cycle, followed by 30 cycles with an annealing temperature of 50°C, and a 10 minute extension period at 72°C.

Fragment lengths were measured in comparison to the GeneScan™ LIZ® 600 Size Standard v. 2.0 (Life technologies) using an Applied Biosystems 3730XL (Life Technologies) at the DNA Sequencing Facility at the University of California Berkeley, and scored using the Microsatellite Plug-in for Geneious 5.6.2 (Drummon *et al.* 2012). The number of alleles per locus (*k*), averaged observed (Ho) and expected (He) heterozygosity, deviations from Hardy-Weinberg equilibrium (HWE), and presence of linkage disequilibrium (LD) between loci were examined using GenePop 4.2 (Raymond & Rousset 1995; Rousset 2008).

Results

Next-Generation Sequencing Results

Our Illumina sequencing run for *T. pallidus* resulted in over 99 million 100 base pair reads and our Illumina sequencing run for *C. juglandicola* resulted in over 170 million 100 base pair reads. Using the *de novo* genome assembly program Velvet (Zerbino & Birney 2008) we constructed 65,535 contigs with an average length of 834.2 base pairs and an average coverage of 8.0X for *T. pallidus*, 474,388 contigs with an average length of 2,573 base pairs and an average coverage of 11.2X for *C. juglandicola*.

Comparison of iMSAT to other methods for identifying STRs

Using MSATCOMMANDER (Faircloth 2008) and Phobos (Mayer 2010) we identified 18,525 and 21,860 STRs for *T. pallidus* (Table 3.2) and 187,270 and 100,290 STRs for *C. juglandicola* (Table 3.3). Using our novel python program iMSAT (https://sourceforge.net/projects/imsat/), we found 9,119 candidate polymorphic STRs for *T. pallidus* (Table 3.2) and 2,378 candidate polymorphic STRs for *C. juglandicola* (Table 3.3). For *T. pallidus* di-nucleotide STRs were the most abundant type identified by all three methods in between 55 and 65%, tri-nucleotide STRs represent between 31 and 38% and tetra- and penta-nucleotide STRs between 4 and 6% combined. For *C. juglandicola* di-nucleotide STRs were again the most abundant type identified by all three methods (82-93%) however for this species, tri-nucleotide STRs were rare (4.6-17%) while tetra- and penta-nucleotide STRs were extremely rare (0-3%).

Amplification of tri-nucleotide STRs in T. pallidus

Of the selected 20 STRs from our output of candidate polymorphic tri-nucleotide STRs we consistently amplified 17 of them with standard PCR protocols. Two of these markers, TpMSAT3 and TpMSAT6 were excluded from the analysis because they displayed repeat patterns not consistent with tri-nucleotide STRs. DNA sequences for the repeat region of each STR marker used in this study were uploaded to GenBank (Accession #'s KC477413-KC477427) and their characteristics were summarized in Table 3.4.

Characteristics of STR markers in T. pallidus

Allelic diversity ranged from three alleles per locus for TpMSAT05 to nine for TpMSAT11 and TpMSAT14 (Table 3.4). Measures of averaged heterozygosity ranged from 0.21 to 0.54 for Ho and 0.33 to 0.54 for He (Table 3.4). One locus, TpMSAT05, exhibited a marginally significant deviation from Hardy-Weinberg Equilibrium (HWE) ($\chi^2 = 16.44$, DF = 8, P = 0.04), though this deviation was not significant after Bonferroni's correction for multiple comparisons (corrected $\alpha = 0.013$). Another locus, TpMSAT13, exhibited a highly significant deviation from HWE ($\chi^2 = 40.23$, DF = 8, P = 0.002), which was still significant after Bonferroni's correction (corrected $\alpha = 0.006$). Linkage disequilibrium was not observed between any of the STR markers.

Amplification of tri-nucleotide STRs in C. juglandicola

We selected 20 STRs from our output of candidate polymorphic tri-nucleotide STRs and we were able to consistently amplify 16 of them with standard PCR protocols. Of the 16 markers all but three were found to be polymorphic in our sample populations. One of these markers, CjMSAT12 was excluded from the analysis because it displayed fragment length polymorphisms outside of its expected range. DNA sequences for the repeat region of each STR marker used in this study were uploaded to GenBank (Accession #'s KJ939575-KJ939587) and their characteristics were summarized in Table 3.4.

Characteristics of STR markers in C. juglandicola

Allelic diversity for polymorphic loci ranged from two alleles per locus for CjMSAT01, CjMSAT03, CjMSAT08, CjMSAT09, CjMSAT16, and CjMSAT19 to seven for CjMSAT13. Measures of averaged heterozygosity ranged from 0.08 to 0.15 for Ho and 0.08 to 0.17 for He (Table 3.4). No locus displayed deviations from HWE, and their was no evidence of linkage disequilibrium observed between any of the STR markers.

Discussion

The genomic revolution sparked by the advent of NGS is well underway, and its low per base pair cost and high number of sequence reads yields many benefits and tools (Silva *et al.* 2013), including the rapid development of polymorphic markers for population genetic studies. Our pipeline involving iMSAT identified polymorphic STRs from two simultaneously obtained sequencing reads. The output of iMSAT facilitates the design of primers for population-level studies, reducing the time and expense associated with the production of STRs.

Potential benefits and limitations

The iMSAT program presented in this paper represents a significant improvement over existing techniques of using NGS technologies to identify and develop STR markers. The use of NGS technologies has become a standard for developing STR markers (e.g. Gardner *et al.* 2011; Zalapa *et al.* 2012), and has saved the laborious steps associated with plasmid cloning (see Andrés & Bogdanowicz 2011 for detailed steps for traditional and NGS methods). However, a current roadblock in STR development using NGS technologies is that candidate markers must still be tested to identify whether or not they represent polymorphic regions. Given the large numbers of candidate markers identified by existing software packages (Tables 3.2 and 3.3) selecting candidate loci and validating their polymorphism with PCR can be both expensive and time consuming. In two recent studies that make use of NGS technologies and existing STR software programs to identify candidate markers, PCR screening was used to examine 48 (McEwen *et al.* 2011) and 342 (Jun *et al.* 2012) candidate markers, but only 11 (23%) and 26 (0.76%) of those markers, respectively, were used in the subsequent studies. Whether these relatively low rates of success in the development of effective markers were due to problems with PCR amplification or to fixation of the markers once amplified is unclear. However, for comparison, our approach resulted in 17 of the 20 candidate polymorphic STRs amplifying, and 15 of the 17 (or 75% of the original 20) proving to be polymorphic for *T. pallidus* and thus useful for population genetic research. Similarly, 16 out of 20 candidates amplified consistently and 12 of the 16 (or 60% of the original 20) proved to be polymorphic for *C. juglandicola*.

In addition, our program adds virtually no costs to the overall production of STR markers, as an additional Illumina Sequencing Library, for example, can be produced by a third party for as little as $200 USD (quote from the Functional Genomics Laboratory at the University of California Berkeley, June 2014). Using freely available and commonly used software in conjunction with the program that we have developed, iMSAT, a list of STR markers that are polymorphic can be generated in a fast and cost-effective manner. While we recovered far fewer potential STR markers using our iMSAT program than found using other existing programs, we feel that the added benefit of knowing that these candidate markers are most likely polymorphic greatly outweighs the reduction in numbers of potential markers. Most studies based on STRs continue to make use of a relatively small number of markers (10-50) and there may not be a need for developing upwards of 12,000 STR markers that is possible using NGS technologies. Finally, although we have only validated this approach using Illumina Sequencing, our software program is also compatible with 454 Sequencing (Roche Diagnostics). The 'novelty' of our approach is to use the polymorphism data provided by the raw sequence reads themselves to identify candidate STR markers, and the program takes advantage of the output from existing software tools (Zerbino & Birney 2008; Li & Durbin 2009; Li *et al.* 2009) which can be applied to both Illumina and 454 Sequencing runs.

A similar approach to screening NGS sequence results for polymorphic regions before STR development has previously been presented by Hoffman and Nichols (2011). These authors also pooled DNA extracts to create a single sequencing library for 454 sequencing, re-mapped the individual sequence reads to their *de novo* assembly, and targeted STR repeats that appeared polymorphic. While similar in that both approaches perform *in silico* polymorphism detection, ours has the advantages that a) the use of Illumina sequencing offers lower cost per base pair and a greater number of total base pairs recovered, and b) by using barcoded libraries we were able to assign sequence reads to both of our populations of *T. pallidus* and *C. juglandicola* with only a single run each. This second advantage was particularly valuable, as it allowed us to identify markers that not only were likely to be polymorphic, but whose polymorphism could also be

characterized as either within and/or between populations. This greatly increases the utility of the data in generating useful STR markers, and may in part explain the greater rate of success we observed in isolating polymorphic markers.

Comparison of results with other species of insects

Recently, the availability of published genomes from a diversity of insect species has allowed for comparative genomic analyses, and of interest to this study, examinations into the diversity and distribution of STR motifs. One study conducted by Behura and Severson (2012) compared coding sequences from 25 species of insects representing five different orders, and in contrast to our findings for *T. pallidus* and *C. juglandicola*, found that tri-nucleotide repeats were the most common repeat type across the insect taxa. Their results may be inherently biased towards recovery of tri-nucleotide repeats, however, because of their focus on coding regions of DNA where single or double base pair insertions/deletions are unlikely (Li *et al.* 2002). Another recent study (Pannebakker *et al.* 2010) examined both coding and non-coding regions, compared published whole genome sequence data from 12 species of insects representing six orders, and found that while most species had predominantly di- or tri-nucleotide repeats, no one type was dominant – even congeners differed in which type of repeats were most abundant. The most dramatic example of this was the difference found between *Drosophila simulans* and *D. melanogaster*. While *D. simulans* had relatively equal proportions of di-, and tri-nucleotides as the most abundant repeat types, penta-nucleotide repeats were the most abundant for *D. melanogaster* and more than twice as abundant as any other repeat type. Interestingly, these authors found that STRs were more common among the Hymenoptera and represented a higher percentage of the genome than in any of the other orders of insect examined. The Hymenoptera also differed from other orders in that di-nucleotide repeats were the most abundant type of repeat – between 2 and 5 times more frequent than tri-nucleotide repeats. For the one species of aphid, *Acyrthosiphon pisum,* that was included in their study STRs were about half as abundant as in the species of Hymenoptera examined. Contrary to our results for *C. juglandicola,* they also found that tri-nucleotide repeats were the most abundant type of repeat unit.

Conclusions

We have developed a novel approach for using NGS technologies in conjunction with several popular software packages to identify polymorphic STRs. This approach allowed us to rapidly and cost-effectively develop 15 polymorphic STRs for *T. pallidus* and 12 polymorphic STRs for *C. juglandicola*

Acknowledgements

Funding for this study was provided by an EPA-STAR grant. This work could not have been completed without the technical assistance of J Lozier, B Ort, and S Ramirez. N Tsutsui provided access to the Computational Genomics Resource Laboratory at the University of California Berkeley where *de novo* genome assembly was performed. I would like to thank all of our collaborators who have aided in the collection of *T. pallidus* and *C. juglandicola* individuals, N. Mills, G. Roderick, and R. Nielsen as well as two anonymous reviewers whose comments and suggestions greatly improved the manuscript.

Chapter 3. References

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Table 3.1. – Source populations of *T. pallidus* and *C. juglandicola* used in this study including the number of females genotyped (N), averaged observed (Ho), and expected (He) heterozygosity.

Pop	Location	Host	Collector	Date	N	Ho	He
T. pallidus							
J0029	Bethel, OR	*M. coryli*	J Andersen and C Hedstrom	24vi2010	6	0.544	0.537
J0030	McMinnville, OR	*M. coryli*	J Andersen	24vi2010	6	0.208	0.412
J0001	Durham, CA	*C. juglandicola*	N Mills	06vii2006	12	0.311	0.328
J0008	Tulare, CA	*C. juglandicola*	N Mills	17ix2006	15	0.271	0.373
J0069	Upper Lake, CA	*C. juglandicola*	R Elkins	10ix2010	11	0.312	0.385
J0178	Yuba City, CA	*P. juglandis*	J Andersen	27ix2011	7	0.242	0.360
J0179	Escalon, CA	*C. juglandicola*	J Andersen and M Labbé	05vi2012	12	0.344	0.354
J0188	Newark, CA	*C. juglandicola*	J Andersen and M Labbé	30viii2012	10	0.347	0.384
J0163	Tehran, Iran	*C. juglandicola*	P Starý	24iii2004	12	0.321	0.381
C. juglandicola							
A0046	Modesto, CA	Walnut	J Andersen and K Anderson	7vii2010	9	0.103	0.100
A0052	Linden, CA	Walnut	J Andersen	10vii2010	8	0.112	0.128
A0073	Upper Lake, CA	Walnut	J Andersren and M Labbé	13ix2010	9	0.151	0.165
A0164	Parnac, France	Walnut	J Andersen and M Labbé	2vi2011	20	0.068	0.082

Table 3.2 – STR results from *Trioxys pallidus* comparing the total numbers of discovered repeats for each pattern type (di, tri, tetra, or penta) using Phobos, MSATCOMMANDER, and iMSAT. The total numbers of repeats for each pattern are summed, and presented as a percentage of total repeats found using each software program

	A) Phobos				B) MSATCOMMANDER				C) iMSAT			
repeats	di	tri	tetra	penta	di	tri	tetra	penta	di	tri	tetra	penta
5	4132	1612	262	38	5338	3496	657	44	772	500	93	18
6	3737	3317	683	38	2090	1898	218	4	1718	837	145	8
7	1751	1788	229	6	1243	980	59	2	1181	717	80	1
8	1104	958	64	2	762	417	28	1	765	426	29	1
9	616	379	29	1	411	151	10	0	410	178	9	0
10	355	133	12	0	240	46	9	0	228	72	1	0
11	194	43	8	0	134	19	2	0	177	41	2	0
12	105	20	3	0	60	22	0	0	129	17	1	0
13	52	18	0	0	29	4	0	0	92	5	0	0
14	19	5	1	0	13	10	1	0	64	9	0	0
15	15	9	0	0	15	5	6	0	65	5	0	0
16	7	7	8	0	3	3	2	0	46	1	0	0
17	3	4	0	0	1	4	0	0	51	5	0	0
18	1	3	1	0	5	7	0	0	20	0	0	0
19	5	6	0	0	3	16	0	0	39	0	0	0
20	3	17	0	0	1	34	0	0	36	0	0	0
21+	16	41	0	0	16	7	0	0	124	1	0	0
SUM	12115	8360	1300	85	10363	7119	992	51	5917	2814	360	28
Percent	55.4	38.2	5.9	0.3	55.9	38.4	5.4	0.3	64.9	30.9	3.9	0.3

Table 3.3 – STR results from *Chromaphis juglandicola* comparing the total numbers of discovered repeats for each pattern type (di, tri, tetra, or penta) using Phobos, MSATCOMMANDER, and iMSAT. The total numbers of repeats for each pattern are summed, and presented as a percentage of total repeats found using each software program

	A) Phobos				B) MSATCOMMANDER				C) iMSAT			
repeats	di	tri	tetra	penta	di	tri	tetra	penta	di	tri	tetra	penta
5	21729	7100	347	29	35052	16556	890	33	9	8	3	0
6	12739	4282	123	1	22177	9949	326	4	39	7	14	0
7	8973	2628	37	1	16641	5961	133	3	63	12	13	0
8	7009	1546	12	1	13107	3231	65	2	149	30	11	0
9	5416	848	6	0	9805	1580	38	3	275	25	4	0
10	3860	389	6	1	6850	754	26	0	344	12	10	0
11	2746	193	2	0	4844	382	11	0	260	6	5	0
12	1890	97	2	0	3282	173	4	0	244	2	2	0
13	1283	39	2	0	2403	95	9	0	164	2	4	0
14	937	14	2	0	1875	50	9	0	133	5	0	0
15	793	12	1	0	1518	32	7	0	100	1	0	0
16	709	3	1	0	1433	14	5	0	81	0	0	0
17	608	4	0	0	1205	9	1	0	84	0	0	0
18	577	3	0	0	1283	8	0	0	53	0	0	0
19	626	2	0	0	1228	10	0	0	44	0	0	0
20	604	5	0	0	1211	2	0	0	35	0	0	0
21+	12028	24	0	0	16100	18	0	0	125	0	0	0
SUM	82527	17189	541	33	146877	38824	1524	45	2202	110	66	0
Percent	82.3	17.1	0.5	0.03	78.4	20.7	0.8	0.02	92.6	4.6	2.8	0

Table 3.4 – Characteristics of the 15 and 12 polymorphic STRs isolated from *T. pallidus* and *C. juglandicola,* respectively, including fluorescent label, sequence information, repeat motif, fragment lengths of observed alleles, annealing temperature in degrees Celsius (T_A), number of observed alleles (N_A), P values from Hardy-Weinberg Equilibrium statistics (P_{HWE}), and GenBank accession numbers. !

Locus	F - Primer sequence	R – Primer sequence	Repeat motif	Fragment lengths	T_A	N_A	P_{HWE}	GenBank Accession
T. pallidus								
TpMSAT01	6FAM - TATCCCCGCCGCGAATAGAA	GTTTGGCGCACGGCTAAAAG	$(ATC)_{14-18}$	366 – 378	57	5	0.260	KC477413
TpMSAT02	VIC -TGCACCACAATCTGGACCAC	GGTTGCCAACAGACGAGGAA	$(ATC)_{6-20}$	345 – 387	57	7	0.918	KC477414
TpMSAT04	6FAM - ACATGTGTCGCCTCAGCAAG	ACGAGAGAGACCCAAGCACA	$(CGA)_{4-10}$	475 – 493	57	7	0.742	KC477415
TpMSAT05	6FAM - AAAGGAGCTCGTTGTCCGTG	AGCTAAAAAGCGCGAAACCA	$(TGA)_{15-18}$	330 – 336	57	3	0.037	KC477416
TpMSAT07	GCTGCTTCGTTCAATGCCAC	VIC - CTGTGGCTTGACTCCGTCTG	$(CAG)_{4-19}$	322 – 370	57	6	0.324	KC477417
TpMSAT08	NED - TGCTTTGATGCATCCTGGGT	GCCATTCCCCCAAGTGTAGC	$(GAC)_{5-10}$	305 – 320	57	5	0.808	KC477418
TpMSAT09	TGTGGGTGCTTGTGTCACTG	PET - ACAACAACCCCGCGTTACAA	$(TAC)_{3-9}$	294 – 312	57	5	0.066	KC477419
TpMSAT10	VIC - GCCTCAAACAAGTTGCCGTG	TCTGCGAGGGGGACATTGAT	$(GCT)_{2-8}$	396 – 414	57	7	0.093	KC477420
TpMSAT11	PET - ACATCAACGCCGACAAACGA	CGCACAAACCGACGATGATG	$(TCA)_{4-10}$	300 – 336	50	7	0.480	KC477421
TpMSAT12	PET - AAATGCCGGCCTAGAAACGG	CTTACCTGTCGGTGCTGTCG	$(AAC)_{5-9}$	255 – 267	57	5	0.478	KC477422
TpMSAT13	NED - CAGCGCATCGTCAGGATCAA	CTCTTCCACCTCCAGCCAGA	$(TCA)_{3-16}$	422 – 461	57	8	0.002*	KC477423
TpMSAT14	6FAM - CCTTGCATACGCTTGCACAC	ATAGCCAAGTCACAGCTGGC	$(AAG)_{3-11}$	313 – 340	57	9	0.147	KC477424
TpMSAT16	6FAM - ACTGCTCGGCACTTGAACAG	AAACCACCATCGTCGTCGTC	$(TGA)_{12-16}$	317 – 329	57	5	0.273	KC477425
TpMSAT17	VIC - GCTTGCGTCTCAGCATTCTC	TGCCAATTATCGCCGCATCA	$(ATT)_{6-15}$	340 – 367	57	6	0.940	KC477426
TpMSAT19	GCTCCCAGAGAAATTGGTGG	VIC - TTGGGAATTAAAAGTGTTGGCT	$(GAA)_{4-13}$	260 – 287	57	6	0.164	KC477427
C. juglandicola								
CjMSAT01	6FAM - TTCACGGGAACGCCATGAAG	TTGTCCGTGAACCACACGTC	$(TAA)_{10-11}$	210 – 213	50	2	NA	KJ939575
CjMSAT02	NED - CGTCGTCACCAACAGTTTC	TGAACAGTTTAAATGACGTTGATG	$(CAA)_{9-16}$	375 – 396	50	5	0.090	KJ939576
CjMSAT03	PET - TTTCCACGTCCCGTAGTCCA	TTGGACATCACACACACGCA	$(TAC)_{11-12}$	374 – 377	50	2	NA	KJ939577
CjMSAT04	VIC - TGCATTGTGCGTGTGTTTCC	TAACAACACGCCGGTAAGCG	$(TAC)_{18-21}$	347 – 356	50	4	1	KJ939578
CjMSAT05	6FAM - AGAACTTTACGACCCGTGCC	GTTCAACTCGGACCACTCGG	$(ATA)_{10-12}$	291 – 297	50	3	1	KJ939579
CjMSAT08	NED - GCGCGCGTCTCTATCTTTCT	CGTCGATCCTGTCAAAGCGT	$(TAA)_{0-15}$	239 – 284	57	2	NA	KJ939580
CjMSAT09	PET - ACGCGTTTGTCGTCTAGCTC	CGAAACGCGGTGTCTTTGTG	$(TAA)_{9-10}$	276 – 279	50	2	NA	KJ939581
CjMSAT13	PET - CGCCGCCATGATTAAGTCCA	ACAAGCCGTTCCAGTTGGTC	$(CGT)_{10-18}$	264 – 288	50	7	NA	KJ939583
CjMSAT14	VIC - TTATGTGCCGGCAGTTACGG	CGCCGTCGTATTACGTGTCA	$(ATT)_{10-13}$	460 – 469	50	3	0.247	KJ939584
CjMSAT16	6FAM - GGGTACAGCGTTGAGACGTG	TGAACGATTTTCGTCCGCGA	$(ATA)_{7-8}$	367 – 370	57	2	NA	KJ939585
CjMSAT18	NED - TGTGAACTAGCTGCGGGGTA	CACTCTCTGCGCGTTCCATT	$(ATT)_{10-12}$	320 – 326	50	3	1	KJ939586
CjMSAT19	PET - ACCAAGTGCATGGGTACAGC	TTCGACCAGCTGACCGTACT	$(TAC)_{14-15}$	318 - 321	50	2	NA	KJ939587

* indicates a significant deviation from HWE after applying Bonferroni's correction for multiple-comparison

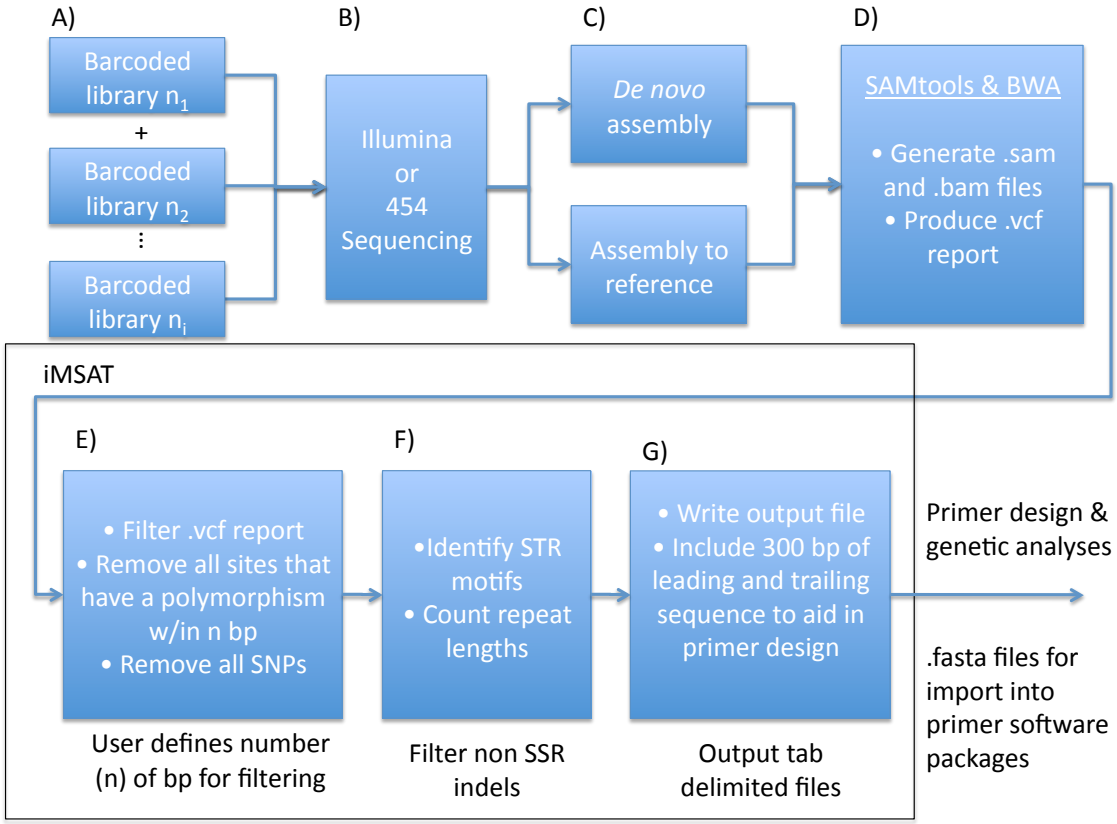

Figure 3.1 – iMSAT workflow diagram. Before using iMSAT, barcoded NGS sequencing libraries are produced (A) and sequenced (B) and either used to create a *de novo* assembly or to align an available reference genome (C). Then using SAMtools and BWA the individual sequence reads are used to create a polymorphism report (D) that includs the location of the polymorphic loci, type (SNP or INDEL), and other quality statistics. iMSAT then uses the output and the alignment file to filter the polymorphism data based on a user specified number of base pairs (E), identifies the STR motifs and the number of repeats (F), and outputs separate .fasta files for each candidate locus that can be used with primer design software (G).

CHAPTER 4: MOLECULAR EVIDENCE OF CRYPTIC SPECIES AND HYBRIDIZATION WITHIN THE *TRIOXYS PALLIDUS* SPECIES COMPLEX

Abstract

Cryptic species complexes have been an important problem in our understanding of biodiversity and the evolution of host ranges. In addition, they have been of particular importance in the use of natural enemies for control of insect pests and weeds because they have delayed implementation and influenced the success of biological control introductions and continue to present safety concerns through potential non-target impacts. In this context, host-associated strains of the parasitoid wasp *Trioxys pallidus* (Haliday) (Hymenoptera: Braconidae) were successfully introduced to the western United States for the biological control of aphids in walnut and filbert orchard systems. Here we examine whether these host-associated strains represent cryptic species, and whether hybridization between them could be responsible for recent breakdowns in the biological control services present in each system. We collected individuals identified as *T. pallidus* from their native and introduced ranges and then reconstructed phylogenetic relationships using the mitochondrial locus cytochrome oxidase I and performed genotyping analyses using 15 polymorphic microsatellite loci. We found that *T. pallidus* is not monophyletic as currently described, and found evidence for the presence of at least two cryptic species. When we compared these results to patterns of host and biogeographic associations we found that one of these cryptic species appeared to be a specialist, while the second species appeared to be a generalist with a broad distribution in the Palearctic region. We found little evidence of hybridization between these cryptic species, and thus, that hybridization is likely not responsible for recent breakdowns of biological control services in the western United States.

Introduction

What role diet breadth plays in shaping the evolutionary trajectory of species has been a longstanding question in the study of evolutionary and co-evolutionary biology (Ehrlich & Raven 1964; Jaenike 1990). In particular, it has been well studied among insect herbivores where host plant ranges can drive patterns of biodiversity (Janz *et al*. 2006; Janz & Nylin 2008). One of the key results from this research is that given the wide diversity of plant life, most insect herbivores appear to be restricted to a relatively narrow subset of host plant species (Bernays & Graham 1988; Futuyma & Moreno 1988; Nosil 2002). A prominent explanation for this diet specialization is the "Jack of all trades, master of none" hypothesis which predicts that a wide host range comes at the cost of reduced efficiency in the use of any particular host (Krasnov *et al*. 2004; Straub *et al*. 2011). It is currently unclear, however, whether as a result of these tradeoffs lineages become more (Kelley & Farrell 1998) or less (Stireman 2005) specialized through time as recent work has shown that host range evolution may be a dynamic process (Janz *et al*. 2001; Nosil 2002; Nosil & Mooers 2005; Winkler & Mitter 2008). As a further complication, it can be difficult to determine whether an organism is a specialist or a generalist because, for many species, ecological factors are often more important in determining host range than evolutionary factors (Hoffmeister 1992; Stireman & Singer 2003; Straub *et al*. 2011). In addition, evolutionary lineages likely oscillate between transient generalist and longer specialist phases (Nylin *et al*. 2014), and populations of

generalists may in fact represent locally adapted cryptic species complexes (Condon & Steck 1997; Nason *et al*. 2002; Moldo *et al*. 2003; Stireman *et al*. 2005; Smith *et al*. 2006).

Cryptic species complexes have often presented problems for the use of natural enemies in the context of biological pest and weed control, as a failure to detect their presence has resulted in delays in implementing and achieving successful control (DeBach 1960; Cox & Williams 1981; Room 1990; Gibson *et al*. 2005; Hemachandra *et al*. 2005) and created safety concerns due to the potential for non-target impacts (Paynter *et al*. 2008; Mason *et al*. 2014). Whether generalist or specialist natural enemies are more effective in providing biological control services (Chang & Kareiva 1999; Symondson *et al*. 2002) has been debated in the literature with compelling arguments for the use of either generalists (e.g. Murdoch *et al*. 1985) or specialists (e.g. Howarth 2000; Barratt *et al*. 2010). However, understanding the tradeoffs in using generalist or specialist natural enemies assumes an accurate knowledge of host-associations and species boundaries, and the presence of unidentified cryptic species complexes makes this impossible. Correctly identifying species boundaries may also aid in our understanding of how host range expansions and/or contractions influence the sustainability and non-target risks associated with biological pest control, as the phenotypic plasticity required for a shift in host range has been noted to be a major driver of speciation and diversification (West-Eberhard 2003; Nylin & Janz 2009; Dennis *et al*. 2011), and may play an important role in the evolution of natural enemies (Henry *et al*. 2010; Zepeda-Paulo *et al*. 2013).

Host-associations can also be influenced by hybridization events (Feder *et al*. 2003), but what role hybridization plays in the sustainability of biological control programs is unclear (Havill *et al*. 2012). On the one hand, hybridization events between closely related specialist herbivores (Bean *et al*. 2013) and target weeds (Blair *et al*. 2008; Williams *et al*. 2014) have resulted in "hybrid breakdown" (i.e. reduced fitness for the insect herbivores, or increased susceptibility for the target weeds). On the other hand, hybridization between insect herbivores has also been shown to result in "hybrid vigor" through greater offspring fitness compared to parents (Szűcs *et al*. 2012). Hybridization can also lead to a dramatic shift in the relative abundance of individuals in a population that possess one of the "parental" genotypes (Yara *et al*. 2007, 2010; Withers *et al*. 2011). In the context of classical biological control, where a non-native pest is re-united with a natural enemy from its region of origin, one practice that may have increased the rates of hybridization is the purposeful introduction of individuals from multiple source locations and/or hosts. Individuals from different sources, often referred to as strains (i.e. biotypes or ecotypes; Clarke & Walter 1995), likely represented populations that were either at an early point of incipient speciation (Dres & Mallet 2002), or even cryptic species that were specialized on different hosts (e.g. Smith *et al*. 2006; Phillips *et al*. 2008; Desneux *et al*. 2009; Heraty 2009; Muirhead *et al*. 2012; Hamback *et al*. 2013).

The biological control programs for walnut aphid, *Chromaphis juglandicola* (Kaltenbach) (Hemiptera: Aphididae), in California and for filbert aphid, *Myzocallis coryli* (Goeze) (Hemiptera: Aphididae), in Oregon provide a valuable study system in which to examine whether host-associated strains of natural enemies are in fact cryptic

species, and to consider the role of host range evolution and effects of hybridization on the sustainability of biological control services. In both nut crop systems (walnuts and filberts) host specific strains of the parasitoid wasp *Trioxys pallidus* (Haliday) (Hymenoptera: Braconidae) were deliberately introduced and resulted in effective suppression of the abundance of their target aphid species (Frazer & Van Den Bosch 1973; van den Bosch *et al*. 1979; Messing & AliNiazee 1988, 1989). For the walnut aphid program in California, *T. pallidus* was originally introduced from southern France and while effective in the southern coastal region (van den Bosch *et al*. 1962), it was a subsequent introduction from Iran that lead to effective suppression of aphid populations in the interior region of the Central Valley (van den Bosch *et al*. 1970). Introductions of *T. pallidus* for filbert aphid in Oregon also originated from France, but included populations from Spain and Italy as well (Messing & AliNiazee 1989). These successful biological control programs have recently shown localized failures in the ability of *T. pallidus* to provide sufficient pest control services, and the reasons for these failures are unknown (Hougardy & Mills 2009; Walton *et al*. 2009). An earlier study had shown that individuals of *T. pallidus* reared from filbert and walnut aphids lacked clear morphological differences and could form viable hybrids in the laboratory, though there were sex ratio differences between the reciprocal crosses (Messing & AliNiazee 1988). Therefore, we suspected that hybridization between individuals of each strain may have played a role in the breakdown of biological control services for these two aphids. Recently, we developed microsatellite markers to examine the population structure of *T. pallidus* in walnut and filbert orchards (Andersen & Mills 2014). Here we take advantage of the availability of these markers, and use a combination of mitochondrial DNA sequencing and microsatellite genotyping to analyze current populations of *T. pallidus* from both its native and introduced ranges. Our objectives in this analysis were to determine 1) whether the strains of *T. pallidus* represent a cryptic species complex, 2) whether different patterns of host association occur among *T. pallidus* strains, and 3) to what extent hybridization has occurred among strains of *T. pallidus*. Finally, we comment on how host range evolution and hybridization may influence the sustainability of these and other biological control programs.

Methods

Sampling Locations and Methods

We sampled walnut and filbert orchards at a series of locations along a transect from the San Joaquin Valley of California in the south (where we expected populations to be primarily composed of pure Iranian walnut strain of *T. pallidus*) through to the Willamette Valley of Oregon in the north (where we expected populations to be composed of pure European filbert strain of *T. pallidus*). Due to the uncertainty as to where, or if, a hybrid zone would exist between these two strains of *T. pallidus* introduced to western North America, we prioritized our sampling in California and Oregon to include individuals from as many different locations as possible rather than collecting large numbers of individuals from a small number of locations. When possible, however, larger sample sizes were collected. At each sampling location, individuals identified as *T. pallidus* were collected either by aspirating adults, or by collecting mummified aphids of *C. juglandicola*, *Panaphis juglandis* (Goeze) (a second aphid species on walnut), or *M. coryli* by removing leaves or leaflets and cutting out a

small section of leaf material to allow individual mummified aphids to be placed into glass vials (9.5 mm high, 3 mm diameter) with a foam stopper. These vials were held at room temperature until adults of *T. pallidus* had emerged from the aphid mummies. Once emerged, or aspirated, all adult *T. pallidus* were stored in 95% ethanol at -20 °C for molecular analysis.

In addition, we collected individuals identified as *T. pallidus* from locations representative of the source populations used for the original introductions into the western United States. These included locations in southern France, northern Italy, and samples provided by Dr. Eshan Rakhshani collected from walnut trees in two locations in Iran. Adults of *T. pallidus* were similarly stored in 95% ethanol at -20°C for molecular analysis. Collection information for all individuals of *T. pallidus* from their native and introduced ranges can be found in Table 4.1, and collection locations are shown in Appendix Figure 1.

DNA Extraction

DNA extraction was performed for 190 females of *T. pallidus* using the Qiagen DNeasy kit (Qiagen Corp.) following the manufacturers protocols, except for as noted below. Individual females were removed from ethanol storage and allowed to air dry for 5 min before being ground using a mortar and pestle and incubated for no less than 12 h in the Cell Lysing Buffer at 55 °C. Following incubation, the extract was centrifuged at 13,300 RPMs for 3 min, or until residual insect remains formed a tight pellet. The extract was then allowed to rest for 5 min before the supernate was transferred to a clean microcentrifuge tube. We then followed the standard Qiagen protocol until the DNA elution step, which was based on 100 µl of buffer AE.

mtDNA Sequencing

Standard PCR protocols were used to amplify a portion of the mitochondrial gene Cytochrome-Oxidase I (COI) with the primer pair LCO and HCO (Folmer *et al*. 1994). PCR reactions were carried out on a BioRad Dyad programmable thermocycler (BioRad Laboratires, Inc.) following conditions presented by Hebert *et al*. (2003) using Promega GoTaq DNA polymerase and buffers (Promega) with the following conditions; 5 µl of Promega GoTaq buffer, 0.5 µl of dNTP (Promega), 0.5 µl of both forward and reverse primers, 0.2 µl of Promega GoTaq taq polymerase, 1 µl of DNA, and HPLC purified H_20 was then added to bring the final volume to 25 µl. PCR products were held at 17 °C before being visualized on a 1.5% agarose gel. DNA sequencing of both forward and reverse fragments was performed at the University of California Berkeley DNA Sequencing Facility, and sequence results were visualized and edited using Geneious Pro v. 5.6.2 (Drummond *et al*. 2012). Individual DNA sequences are available on GenBank (accession numbers KM973216 - KM973401 & KR074103 - KR074105).

mtDNA Analyses

To examine whether strains of *T. pallidus* differed based on mitochondrial sequence diversity, we constructed an alignment of all of our sequenced individuals using

Geneious Pro v. 5.6.2 (Drummond *et al*. 2012). As there were no insertions or deletions present within the sequence fragments, alignment was trivial. To this alignment we added outgroup sequences for several species of closely related braconid parasitoids obtained from GenBank, including *Aphidius funebris* (JX507450), *A. matricariae* (JX507432), *Monoctonus cerasi* (JX507448), *Binodoxys acalephae* (JX507441), *B. centaureae* (JX507447), *B. communis* (FJ798201), *T. curvicaudus* (KM973350), and *T. complanatus* (KJ848479). We also added two ingroup sequences published on GenBank for specimens of *T. pallidus* collected in southern France from *Tuberculatus* sp. (JN620695-6) as well as nine sequences (GCNGRF305-12, GSSHH153-14, GSSHH165-14, GSSHH160-14, GHSSHH166-14, JSHYN764-11, NCCC742-11, NGAAD869-14, SMTPJ5260-14) published through the Barcode of Life Database (BOLD) systems and identified as *T. pallidus* using their BIN algorithm (Ratnasingham and Hebert 2013). The alignment was then visualized and truncated using MacClade v. 4.0.8 (Maddison & Maddison 2005). Phylogenetic analyses were performed using MrBayes v. 3.2.1 x64 (Huelsenbeck 2001) compiled for use with parallel processors, and run through the CIPRES Science Gateway v. 3.1 (Miller *et al*. 2010). Analyses were performed using two independent runs, each with four chains, with a run time of 20 million generations and a burn-in period of 2 million generations. The alignment was divided into three partitions, one for each codon position, and each codon position was analyzed using the GTR+I+G nucleotide model of evolution. To assess whether the runs had converged, we first compared the standard deviation of the two runs, and then visualized the results with the program TRACER (Rambaut & Drummond 2007).

To further examine biogeographic and host-association patterns we then excluded outgroup species from the dataset, and used a network analysis as performed through the software program TCS v 1.21 (Clement *et al*. 2000) with a 95% connection limit. Haplotype groups were then drawn proportional to the number of included sequences using Adobe Illustrator CS2 (Adobe Systems Inc.).

Microsatellite Genotyping

Microsatellite genotyping was performed using the polymorphic microsatellite loci and PCR protocols presented in Andersen and Mills (2014). Briefly, for each individual female, 15 polymorphic microsatellite loci were amplified using PCR. PCR products for up to four loci were pooled, so that no two loci with the same fluorescent label were combined, and the pooled products were genotyped on an Applied Biosystems 3730XL DNA Analyzer at the University of California Berkeley DNA Sequencing Facility. Fragment lengths were then scored using the Microsatellite Plug-in for Geneious Pro v. 5.6.2 (Drummond *et al*. 2012).

Population Structure and Hybridization Analyses

To determine the number of distinct genetic clusters that individuals of *T. pallidus* collected from its native and introduced range could be assigned to, and the number of individuals of hybrid origin, the genotyping results for the 15 polymorphic microsatellite loci were analyzed using the program STRUCTURE (Pritchard *et al*. 2000). Four independent runs each of 10 million generations with a burn-in period of 1 million

generations were processed through the online bioinformatics server BioPortal (http://www.bioportal.uio.no) at the University of Oslo, Norway, for potential cluster numbers (K) of one through six. Based on the results from these analyses, we i) determined the optimal K value based on the approach presented by Evanno *et al*. (2005) using STRUCTURE HARVESTER (Earl & vonHoldt 2012), and ii) determined whether individuals were of hybrid origin based on their probabilities of assignment to genetic clusters (Q). Any individual receiving an assignment of $Q > 0.80$ to any one cluster was classified as representing a "pure" individual of that cluster, while individuals with $Q < 0.80$ were classified as being of hybrid origin (Havill *et al*. 2012).

In addition, we directly estimated the probability of assignment (Z) that an individual was of parental or hybrid origin using the software program NewHybrids (Anderson 2008; Anderson & Thompson 2002). NewHybrids can only estimate values for Z when two potential parent populations are examined; therefore we used the results from our STRUCTURE analysis to conduct pairwise comparisons between all recovered population pairs. While NewHybrids directly estimates the probability of assignment to F1, F2, and backcross categories, we summed the probabilities of assignment for each of these hybrid categories into a single combined hybrid group. We constructed datasets for each pairwise comparison that included all individuals with $Q > 0.8$ for one of the two populations, and all individuals classified as hybrids with $0.8 > Q > 0.25$ for both populations being compared. For each pairwise comparison, we used random starting values and allowed NewHybrids to run for 10 million generations with a burn-in period of 1 million generations using a uniform prior for the estimates of both theta and pi.

Results

mtDNA Analysis

We sequenced a fragment of COI from 189 *T. pallidus* females collected in California (n =125), Oregon (n=30), Europe (n=22), and Iran (n=12). After the addition of published outgroup and ingroup sequences and the truncation of the dataset, the resulting alignment included 207 sequences and 625 basepairs. GenBank accession numbers and mtDNA haplotype information for each individual are presented in Table 4.1. The results from our MrBayes analysis indicated that after 20 million generations, the two independent runs had converged with an average standard deviation of split frequencies between the runs of 0.0418. The majority-rule consensus tree from the MrBayes analysis (Figure 4.1) reconstructed a clade (Clade A) that included all individuals identified as *T. pallidus* as well as the single published sequence for *T. complanatus* with high Bayesian posterior probability support (100% BPP). Based on this reconstruction, *T. pallidus* is not monophyletic as the sequence from *T. complanatus* was found with high support (90% BPP) to be sister to another well supported clade (Clade B; 97% BPP) that includes all of the sequences from specimens of *T. pallidus* reared from filbert aphids in Oregon, all but one of the sequences from specimens of *T. pallidus* reared from filbert aphids in Europe, and all of the sequences published through BOLD Systems collected in Canada. A separate highly supported clade (Clade C; 90% BPP) included all of the sequences from specimens of *T. pallidus* collected from walnut and dusky-veined aphids in California, Europe, and Iran, two published sequences from GenBank for *T. pallidus* reared from *Tuberculatus* sp. in France, four published

sequences from BOLD Systems collected in Norway, and one sequence from an individual reared from filbert aphid in Italy.

The TCS analysis reconstructed two independent networks. One network, labeled "Network 1," included 22 haplotypes, while the second network, labeled "Network 2," included eight haplotypes (Figures 4.2 and 4.3). Network 1 included all of the individuals collected from California and Iran, and all of the published sequences from Norway, as well as the majority of individuals from France and Italy (Figure 4.2). This network also included all individuals reared from *C. juglandicola* and *P. juglandis*, as well as a single individual (J0138b) reared from *M. coryli* (Figure 4.3). Network 2 included the majority of individuals collected from Oregon and all of the published sequences from Canada (Figure 4.2), and only included individuals reared from *M. coryli* or from unknown hosts (Figure 4.3).

Population Structure Analysis

We successfully amplified polymorphic microsatellite loci from 190 females of *T. pallidus* (these represented all of the individuals we sequenced for the mtDNA analysis as well as an additional specimen J0030C from Oregon whose DNA aliquot was exhausted before sequencing of COI was completed). The results from our STRUCTURE analysis indicated that negative log-likelihood values continued to improve with increasing numbers of potential genetic clusters from 2 to 6. However, using STRUCTURE HARVESTER we found that a subdivision that included three clusters was optimal in that it represented the maximal rate of change of the log likelihood scores between STRUCTURE runs of successively increasing K values (i.e. the ΔK statistic from Evanno *et al*. [2005]). The probabilities of assignments for each individual to these three clusters are shown graphically in Figure 4.4 and presented as text in Table 4.1. One genetic cluster (Cluster 1; shown in dark grey) included only individuals collected from filbert orchards in Oregon and one individual collected from a filbert aphid on a roadside tree near Bordeaux, France. A second genetic cluster of *T. pallidus* females (Cluster 2; shown in light grey) included all individuals collected from walnut orchards in California and Iran, as well as one individual collected from a walnut orchard in Oregon. The final genetic cluster (Cluster 3; shown in white) included all individuals collected from walnut aphids in Western Europe, several individuals collected from filbert aphids in Western Europe, two individuals collected from walnut aphids in Berkeley, California, and one individual collected from a walnut orchard in Oregon.

Hybridization Analysis

Using $Q < 80\%$ for any one genetic cluster as a criterion for identifying hybrids in STRUCTURE, only three females of *T. pallidus* were classified as being hybrids. One individual collected in California (J0008c) appears to be a hybrid between individuals from Cluster 2 (Q =77.1%) and Cluster 3 (Q = 20.3%). A second individual collected in Oregon (J0026a) appears to be a hybrid between individuals from Cluster 1 (Q= 20.9%) and Cluster 2 (Q=78.6%). The third individual was collected in Iran (J0161F) and appears to be a hybrid between Cluster 2 (Q = 67.7%) and Cluster 3 (Q=31.1%).

Based on the results from the pair-wise NewHybrids analyses, we confirmed the hybrid status of two of the three females of *T. pallidus* identified with STRUCTURE (Figure 4.5; Table 4.1). Individual J0026a was classified as being of hybrid origin with high probability (Z = 85.5%) in the Cluster 1 and Cluster 2 comparison, and individual J0008c was classified as being a hybrid, though with low probability of assignment (Z = 58.3%) in the Cluster 2 and Cluster 3 comparison. Individual J0161F that was classified as a hybrid by STRUCTURE was not identified as a hybrid by NewHybrids. Six additional individuals received low probabilities of assignment for being of hybrid origin. Two individuals collected from a walnut orchard in Oregon (J0024a & b) included assignment to hybrid classes in the Cluster 2 and Cluster 3 comparison (Z = 53.1% and Z = 44.6%, respectively); one individual reared from a filbert aphid in Italy (J0137a) included assignment to hybrid classes in the Cluster 1 and Cluster 2 comparison (Z = 23.3%); and two additional individuals also from filbert aphids in Italy (J0138A and J0189A) and one individual collected in Berkeley, CA (J0006a) included assignment to hybrid classes in the Cluster 1 and Cluster 3 comparison (Z = 34.7%, Z = 13.3%, and Z = 15.8%, respectively).

Discussion

For insect herbivores, host-associated differentiation is usually represented either by local adaptation (Alstad *et al*. 1980; Alstad & Edmunds 1983; Glynn & Herms 2004; Ruiz-Montoya & Nunez-Farfan 2013; Stotz *et al*. 2013) or by expansion onto novel, but phylogenetically related, plant species (Feder *et al*. 2003; Hamback *et al*. 2013). In contrast, for insect parasitoids, there are far fewer examples of sequential radiation in which parasitoids diversify in response to the plant-associated diversification of their herbivorous hosts (see Abrahamson & Blair 2008). Many studies of specialized parasitoids have found no evidence of host-associated diversification (Baer *et al*. 2004; Lopez-Vaamonde *et al*. 2005; Althoff 2008; Lozier *et al*. 2008; Dickey & Medina 2010, 2011; Bilodeau *et al*. 2013), and differentiation appears to have resulted more frequently from shifts to hosts that represent entirely different families or even orders (Yeates & Greathead 1997; Symonds & Elgar 2013; Tachi 2013). Here we find that suspected host-associated strains of the parasitoid wasp *Trioxys pallidus* that were successfully introduced to the western United States from Europe and Iran for the biological control of walnut and filbert aphids, in their respective crop systems (van den Bosch *et al*. 1970; Messing & AliNiazee 1989), may in fact represent a cryptic species complex with at least two cryptic species based on general patterns of concordance across mitochondrial and nuclear loci (Figure 4.6).

Without an accurate phylogeny for *Trioxys* species we can only speculate as to the direction of evolution of their host-associations. However, we find that one of the two cryptic species in the *T. pallidus* complex (Clade B in Figure 4.1) appears to be a specialist, while the other (Clade C in Figure 4.1) appears to be a generalist as it includes individuals that were reared from two species of aphids on walnut trees (*C. juglandicola* and *P. juglandis*), one individual reared from *M. coryli,* and published sequences from two individuals reared from a species of *Tuberculatus*. The aphid hosts of the *Trioxys* species included in this study are all members of the aphid tribe Panaphidini (Aphididae: Calaphidinae; Kavallieratos *et al*. 2004; Pons *et al*. 2006; Fulbright *et al*. 2007).

Therefore, in contrast to patterns of host-associations seen in many insect parasitoids (e.g. Yeates & Greathead 1997; Symonds & Elgar 2013; Tachi 2013), host-associations in this group are more likely a result of local adaptation rather than shifts to novel hosts. In addition, based on the known host-associations for *T. curvicaudus* and *T. pallidus*, both have been recorded from a number of the same species (e.g. *Eucallipterus* spp., *Tuberculatus* spp., and *Myzcocallis* spp. [Kavallieratos *et al*. 2004; Fulbright *et al*. 2007]), it seems likely that the ancestor of these two *Trioxys* species (Clade A) had a wide host range that included, at least, the above aphid species. The apparent specialization of *T. complanatus* on *Therioaphis trifolii* and of *T. pallidus* Clade B on *Myzocallis* spp., is thus consistent with the specialization as a dead end hypothesis (Kelley and Farrell 1998). However, whether the generalist cryptic species (Clade C) is currently expanding or contracting its host range is unclear.

One curious finding from our analyses was the inclusion in *T. pallidus* Clade B of the five published sequences from individuals collected in British Columbia, Saskatchewan, and Ontario, Canada. Four of these sequences were an exact match to sequences we obtained from individuals reared from *M. coryli* in Oregon and Italy, suggesting that they are descendants from the individuals introduced to the western United States. Native species of *Corylus* (i.e. hazelnuts or filberts in a commercial setting) can be found throughout Canada and the United States (USDA 2015), and while these species are hosts to a diverse assemblage of aphid species, these aphids are exclusively in the tribe Macrosiphini (Blackman & Eastop 1994). The *T. pallidus* cryptic species complex appears to be confined to aphids in the tribe Panaphidini (Kavallieratos *et al*. 2004; Pons *et al*. 2006; Fulbright *et al*. 2007). However, *C. avellana* (i.e. filbert) has been planted as an ornamental in many locations in both Canada and the United States, and it is therefore likely that these sequences belong to individuals that emerged from *M. coryli* using these ornamental plants. In addition, there are several species of *Myzocallis* that can be found on oaks (*Quercus* sp.) and chestnuts (*Castanea* sp.) in North America. Therefore, while the host associations for the individuals collected in Canada are unknown, it is therefore likely that these individuals emerged from either *M. coryli* on ornamental plants or another species of *Myzocallis* on native North American trees.

Hybridization and the Sustainability of Biological Control Services

In the context of classical biological control, until recently, natural enemies were frequently introduced from multiple sources as different "strains" (Clarke & Walter 1995) in the hope that introducing a more diverse pool of individuals would either overcome the genetic bottlenecks associated with founder populations (Hopper *et al*. 1993) or allow the most fit strain to establish (Phillips *et al*. 2008). However, the introduction of distinct genetic lineages that were reproductively isolated in their ancestral range raises the question of whether potential hybrids would be more or less fit than their parents. There has also been recent concern about hybridization between introduced and native species of biological control agents (Havill *et al*. 2012) and that hybridization may facilitiate the the spread of invasive species (Facon *et al*. 2011; Turgeon *et al*. 2011). The two ecologically distinct host strains of *T. pallidus* that we recovered from the western United States in our study had shown evidence of hybrid breakdown in an earlier laboratory study (Messing & AliNiazee 1988). Similarly,

laboratory evidence of hybrid breakdown between Moroccan and European strains of the parasitoid *Microctonus aethiopoides* lead to rejection of the European strain for introduction into New Zealand for control of the clover root weevil in favor of a parthenogenetic strain from Ireland (Goldson *et al*. 2003; Gerard *et al*. 2006). In contrast, intraspecific hybrids of *Psyttalia lounsburyi* did not show differential fitness with respect to parent populations (Benvenuto *et al*. 2012a), and hybrid crosses between different species of *Trichogramma* have shown both hybrid breakdown and hybrid vigor (Benvenuto *et al*. 2012b). While the potential for negative effects of hybridization currently appear to outweigh the positive effects, the outcome of hybridization among ecologically distinct populations of introduced natural enemies may be either taxon specific or related to the degree of genetic separation between populations. Either way, any generalities about the role of hybridization in the context of biological control introductions await a more extensive set of studies that address both the genetic structure and fitness of hybrids from multiple source populations.

With recent localized failures in both the walnut and filbert aphid biological control programs in the western United States (Hougardy & Mills 2009; Walton *et al*. 2009), we expected that hybridization might be a leading cause for the reduction in pest control services. However, based on our analyses, we found that while hybridization between cryptic species of *T. pallidus* occured in both its native and introduced ranges, the rates were far too low to account for the resumption of in-season insecticide use in the western United States. We will continue to monitor whether other genetic factors that are known to affect biological control programs, including genetic bottlenecks (Hufbauer 2002; Hufbauer & Roderick 2005; de Boer 2012; Omondi *et al*. 2014; Seabra *et al*. 2015;) Allee effects (Fauvergue & Hopper 2009; Fauvergue *et al*. 2012) or restricted geneflow (Vaughn & Antolin 1998) may be playing a role in the observed breakdowns in this textbook classical biological control program.

Conclusions

Our study suggests previously identified host-associated strains of the parasitoid wasp *T. pallidus* represent members of a cryptic species complex. The elevation of these strains to species status has previously been proposed (Caltagirone 1985; Clarke and Walter 1995), but no known morphological characters exist to separate these individuals (Messing & AliNiazee 1988). One of these cryptic species (Clade B) appears to be a specialist on *Myzocallis* spp., while the second cryptic species (Clade C) appears to be a more generalist parasitoid of tree-dwelling aphids in the Panaphini. While we found evidence of hybridization between individuals from these cryptic species in both their native and introduced ranges, the low levels we observed are not sufficient to account for the recent failures in the biological control programs in the western United States. Lastly, given the widespread use and importance of *Trioxys* species as biological control agents of aphid species globally (e.g. *T. angelicae* [Mahmoudi *et al*. 2010]; *T. brevicornis* [Starý 1990]; *T. complanatus* [Milne 1997]; *T. curvicaudus* [Zuparko 1983]; *T. indicus* [Singh & Agarwala 1992]; *T. monelliopsis* [Starý & Marsh 1982]; *T. tenuicaudus* [Olkowski *et al*. 1982]), our finding that *T. pallidus* is not monophyletic, as currently described, strengthens the call for a thorough phylogenetic analysis of this important genus of biological control agents (Rakhshani *et al*. 2012).

Acknowledgements

Funding for this study was made possible by an EPA-STAR Fellowship and a Robert van den Bosch Memorial Fellowship. I would like to thank P. Starý and E. Rakhshani for providing specimens of *T. pallidus*, as well as A. Romero and his staff at the Investigación y Technología Agroalimentaria in Tarragona, Spain for their assistance collecting *T. pallidus*. I would also like to thank R. Messing for sharing his Dissertation collection notes; S. Moraglio for organizing locations for collection in Italy; K. Anderson, R. Elkins, C. Hedstrom, A. Lemieux, K. Mace-Hill, L. Morril, S. Renquist, for collecting specimens in California and Oregon, and M. Labbé for her field assistance on two continents. In addition, I would like to thank the University of California Cooperative Extension Specialists; the East Bay Regional Parks Department; the individual walnut and filbert growers who granted us access to their properties; J. Lozier and B. Ort for their technical assistance in conducting the molecular analyses; and N. Mills, R. Nielsen, and G. Roderick for their comments on an earlier draft of this chapter.

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Table 4.1 – Geographic locality information, summary of results from mtDNA, STRUCTURE and pairwise NewHybrid analyses for ingroup and outgroup taxa.

Previously published ingroup and outgroup sequences

Species Name	**Locality**	**Database & Accession**	mtDNA Haplotype
Aphidius funebris	Silwood Park, Ascot, Berkshire, United Kingdom	GenBank JX507450	
A. matricaria	Silwood Park, Ascot, Berkshire, United Kingdom	GenBank JX507432	
Binodoxys acalephae	Silwood Park, Ascot, Berkshire, United Kingdom	GenBank JX507441	
B. communis	China, ex. *Aphis glycines*	GenBank FJ798201	
B. centaurea	Silwood Park, Ascot, Berkshire, United Kingdom	GenBank JX507447	
Monoctonus cerasi	Silwood Park, Ascot, Berkshire, United Kingdom	GenBank JX507448	
Trioxys curvicaudus	Italy: Caluso (7° 53' E; 45° 18' N) 22vi2010 col: J.C. Andersen and M.A. Labbé, ex. *Eucallipterus tiliae*	GenBank KM973350	
T. complanatus	Iran	GenBank KJ848479	
T. pallidus	S.E. France, ex. *Tuberculatus* spp.	GenBank JN620695	JN620695
T. pallidus	S.E. France, ex. *Tuberculatus* spp.	GenBank JN620696	JN620695
Trioxys sp.	Grasslands NP, Saskatchewan Canada	BOLD CNGRF305-12	J0019a
Undet	Ontario, Canada	BOLD JSHYN764-11	JSHYN764-11
Undet	Ontario, Canada	BOLD NCCC742-11	J0019a
Undet	Britich Columbia, Canada	BOLD NGAAD869-14	J0019a
Undet	Ontario, Canada	BOLD SMTPJ5260-14	J0019a
Undet	Rogaland, Norway	BOLD GSSHH153-14	GSSHH153-14
Undet	Rogaland, Norway	BOLD GSSHH160-14	GSSHH160-14
Undet	Telemark, Norway	BOLD GSSHH165-14	GSSHH165-14
Undet	Telemark, Norway	BOLD GSSHH166-14	GSSHH166-14

Specimen information for individuals of *T. pallidus* novel to this study

ID	**GenBank Accession**	**mtDNA Haplotype**	**Structure Assignment**	**NewHybrid 2,1**	**NewHybrid 1,3**	**NewHybrid 3,2**
J0001	United States: California, Durham (121° 50' W; 39° 40' N) 6vii2006 ex: *Chromaphis juglandicola*					
J0001e	KM973216	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0001f	KM973217	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0001g	KM973218	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0001h	KM973219	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0001i	KM973220	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0001j	KM973221	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0001k	KM973222	J0001e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0001l	KM973223	J0001e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0001m	KM973224	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0001n	KM973225	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0001o	KM973226	J0001f	0/0.92/0.08	1/0/0		0/0.01/0.99
J0001p	KM973227	J0001e	0/1/0	1/0/0		0/0.01/0.99

J0002	United States: California, Rumsey 7ix2006 ex: *C. juglandicola*					
J0002a	KM973228	J0001e	0/0.99/0.01	1/0/0		0/0.01/0.99
J0004	United States: California, Cressey (120° 41.63' W; 37° 26.882' N) col: N.J. Mills ex: *C. juglandicola*					
J0004b	KM973229	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0005	United States: California, Goshen (119° 29.503' W; 36° 20.908' N) 12ix2009 ex: *C. juglandicola*					
J0005a	KM973230	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0005b	KM973231	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0006	United States: California, Berkeley (122° 16' W; 37° 52' N) 27ix2007 ex: *C. juglandicola*					
J0006a	KM973232	J0001f	0.04/0/0.96		0/0.16/0.85	0.99/0.01/0
J0006b	KM973233	J0001e	0/0/0.99		0/0.01/0.99	0.99/0.01/0
J0008	United States: California, (36.19, -119.29) 17ix2006 ex: *C. juglandicola*					
J0008a	KM973234	J0001e	0/0.99/0.01	1/0/0		0/0.01/0.99
J0008b	KM973235	J0001e	0.03/0.86/0.1	0.99/0.01/0		0/0.02/0.98
J0008c	KM973236	J0001e	0.03/0.77/0.2			0.05/0.58/0.37
J0008d	KM973237	J0001f	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0008e	KM973238	J0001e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0008f	KM973239	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0008g	KM973240	J0001e	0.01/0.96/0.03	0.99/0.01/0		0/0.01/0.99
J0008h	KM973241	J0001e	0.01/0.99/0	0.99/0.01/0		0/0.01/0.99
J0008i	KM973242	J0001f	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0008j	KM973243	J0001e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0008k	KM973244	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0008l	KM973245	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0008m	KM973246	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0008n	KM973247	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0008o	KM973248	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0009	United States: California, Hanford 12ix2009 Ex: *C. juglandicola*					
J0009b	KM973249	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0010	United States: California, San Juan Bautista 11viii2008 Ex: *C. juglandicola*					
J0010a	KM973250	J0001e	0.01/0.98/0.01	0.99/0.01/0		0/0.01/0.99
J0019	United States: Oregon, Riggs Damm 25vi2008 Ex: *Myzocallis coryli*					
J0019a	KM973251	J0019a	0.95/0.03/0.02	0/0.01/0.99		
J0023	United States: Oregon, Roseburg (123° 27.073' W; 43° 17.88' N) 23vi2010 col: J.C. Andersen and S. Renquist Ex: *M. coryli*					
J0023a	KM973252	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0023b	KM973253	J0023a	0.99/0/0.01	0/0.01/0.99	0.99/0.01/0	
J0024	United States: Oregon, (123° 27.321' W; 43° 17.839' N) 23vi2010 col: J.C. Andersen and S. Renquist Ex: *C. juglandicola*					
J0024a	KM973254	J0024a	0/0.16/0.84		0/0.01/0.99	0.46/0.54/0
J0024b	KM973255	J0001f	0/0.84/0.16	0.99/0.01/0		0/0.45/0.55
J0026	United States: Oregon, Monmouth (123° 16.387' W; 44° 50.57' N) 24vi2010 col: J.C. Andersen and C. Hedstrom Ex: *M. coryli*					
J0026a	KM973256	J0023a	0.79/0.21/0.01	0/0.86/0.15	0.99/0.01/0	
J0026b	KM973257	J0023a	0.99/0/0.01	0/0.01/0.99	0.99/0.01/0	
J0027	United States: Oregon, Bethel (123° 11.666' W; 45° 3.448' N) 24vi2010 col: J.C. Andersen and C. Hedstrom Ex: *M. coryli*					
J0027b	KM973258	J0027b	1/0/0	0/0/1	0.99/0.01/0	
J0028	United States: Oregon, Bethel (123° 11.666' W; 45° 3.448' N) 24vi2010 col: J.C. Andersen and C. Hedstrom Ex: *M. coryli*					
J0028a	KM973259	J0023a	0.98/0.01/0.01	0/0.01/0.99	0.99/0.01/0	
J0028b	KM973260	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	

J0029	United States: Oregon, Bethel (123° 11.666' W; 45° 3.448' N) 24vi2010 col: J.C. Andersen and C. Hedstrom Ex: *M. coryli*					
J0029b	KM973261	J0019a	0.95/0.03/0.02	0/0.01/0.99	0.99/0.01/0	
J0029d	KM973262	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0029e	KM973263	J0019a	0.88/0.01/0.11	0/0.01/0.99	0.97/0.03/0	
J0029f	KM973264	J0029f	0.98/0.01/0.01	0/0.01/0.99	0.99/0.01/0	
J0029g	KM973265	J0029f	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0029h	KM973266	J0029h	0.99/0/0	0/0.01/0.99	0.99/0.01/0	
J0029i	KM973267	J0019a	0.99/0/0.01	0/0.01/0.99	0.99/0.01/0	
J0030	United States: Oregon, McMinnville (123° 17.569' W; 45° 5.731' N) 24vi2010 col: J.C. Andersen Ex: *M. coryli*					
J0030b	KM973268	J0023a	0.97/0.02/0.01	0/0.01/0.99	0.99/0.01/0	
J0030C				1/0/0	0/0.01/0.99	0.99/0.01/0
J0030d	KM973269	J0019a	1/0/0	0/0/1	0.99/0.01/0	
J0030e	KM973270	J0019a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0030f	KM973271	J0030f	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0030g	KM973272	J0023a	1/0/0	0/0/1	0.99/0.01/0	
J0031	United States: Oregon, Sheridan (123° 20.424' W; 45° 6.11' N) 24vi2010 col: J.C. Andersen Ex: *M. coryli*					
J0031A	KM973273	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0031B	KM973274	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0032	United States: Oregon, Forest Grove (123° 8.966' W; 45° 34.028' N) 25vi2010 col: J.C. Andersen and A. Lemieux Ex: *M. coryli*					
J0032A	KM973275	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0032b	KM973276	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0033	United States: Oregon, Forest Grove (123° 8.966' W; 45° 34.028' N) 25vi2010 col: J.C. Andersen and A. Lemieux Ex: *M. coryli*					
J0033a	KM973277	J0023a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0033B	KM973278	J0019a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0034	United States: Oregon, Forest Grove (123° 8.966' W; 45° 34.028' N) 25vi2010 col: J.C. Andersen and A. Lemieux Ex: *M. coryli*					
J0034a	KM973279	J0019a	1/0/0	0/0.01/0.99	0.99/0.01/0	
J0034b	KM973280	J0023a	0.96/0.02/0.02	0/0.01/0.99	0.99/0.01/0	
J0035	United States: California, Hollister (121° 26.106' W; 36° 54.226' N) 6vii2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0035a	KM973281	J0001f	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0035b	KM973282	J0001e	0/0.98/0.02	1/0/0		0/0.01/0.99
J0036	United States: California, San Juan Bautista (121° 27.612' W; 36° 51.049' N) 6vii2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0036a	KM973283	J0001f	0.01/0.99/0	0.99/0.01/0		0/0.01/0.99
J0036b	KM973284	J0001e	0 /0.97/0.03	1/0/0		0/0.01/0.99
J0038	United States: California, Lemoore (119° 45.118' W; 36° 17.8' N) 6vii2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0038a	KM973285	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0039	United States: California, (121° 9.551' W; 37° 29.863' N) 7vii2010 col: J.C. Andersen and K. Anderson Ex: *C. juglandicola*					
J0039a	KM973286	J0001f	0/0.99/0	1/0/0		0/0.01/0.99
J0041	United States: California, (121° 9.551' W; 37° 29.863' N) 7vii2010 col: J.C. Andersen and K. Anderson Ex: *C. juglandicola*					
J0041a	KM973287	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0044	United States: California, (120° 53.133' W; 37° 34.818' N) 7vii2010 col: J.C. Andersen and K. Anderson Ex: *C. juglandicola*					

J0044a	KM973288	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0044b	KM973289	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0045	United States: California, (120° 48.4' W; 37° 38.359' N) 7vii2010 col: J.C. Andersen and K. Anderson Ex: *C. juglandicola*					
J0045a	KM973290	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0046	United States: California, Modesto (120° 51.72' W; 37° 38.326' N) 7vii2010 col: J.C. Andersen and K. Anderson Ex: *C. juglandicola*					
J0046a	KM973291	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0047	United States: California, Chico (122° 1' W; 39° 51' N) 25v2010 col: K. Mace-Hill Ex: *C. juglandicola*					
J0047b	KM973292	J0001e	0/1/0	0.99/0.01/0		0/0.01/0.99
J0048	United States: California, Chico (122° 1' W; 39° 51' N) 11vi2010 col: K. Mace-Hill Ex: *C. juglandicola*					
J0048a	KM973293	J0001e	0/1/0	0.99/0.01/0		0/0.01/0.99
J0049	United States: California, Linden (121° 8' W; 38° 3' N) 10vi2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0049a	KM973294	J0001f	0.01/0.99/0	0.99/0.01/0		0/0.01/0.99
J0049b	KM973295	J0001e	0/1/0	0.99/0.01/0		0/0.01/0.99
J0050	United States: California, Linden (121° 8' W; 38° 3' N) 10vi2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0050a	KM973296	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0050b	KM973297	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0051	United States: California, Linden (121° 8' W; 38° 3' N) 10vi2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0051a	KM973298	J0051a	0/1/0	1/0/0		0/0.01/0.99
J0051b	KM973299	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0053	United States: California, Winters (121° 58' W; 38° 30' N) 7vii2010 col: K. Mace-Hill Ex: *C. juglandicola*					
J0053a	KM973300	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0053b	KM973301	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0054	United States: California, Davis (121° 47' W; 38° 32' N) 2vi2010 col: K. Mace-Hill and L. Morril Ex: *C. juglandicola*					
J0054a	KM973302	J0054a	0/1/0	1/0/0		0/0.01/0.99
J0054b	KM973303	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0055	United States: California, Davis (121° 47' W; 38° 32' N) 2vi2010 col: K. Mace-Hill and L. Morril Ex: *C. juglandicola*					
J0055a	KM973304	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0055b	KM973305	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0057	United States: California, (122° 4.307' W; 38° 57.938' N) 19vii2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0057a	KM973306	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0057b	KM973307	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0058	United States: California, (121° 34.248' W; 38° 53.05' N) 19vii2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0058a	KM973308	J0051a	0.01/0.976/0.014	1/0.01/0		0/0.01/0.99
J0058b	KM973309	J0001f	0.002/0.98/0.018	1/0.01/0		0/0.01/0.99
J0059	United States: California, (121° 36.891' W; 38° 52.347' N) 19vii2010 col: J.C. Andersen Ex: *C. juglandicola*					
J0059a	KM973310	J0001f	0/1/0	1/0.01/0		0/0.01/0.99
J0061	United States: California, (121° 38.154' W; 39° 6.765' N) 19vii2010 col: J.C. Andersen Ex: *C.*					

	juglandicola					
J0061a	KM973311	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0061b	KM973312	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0069	United States: California, (122° 53' W; 39° 10' N) 10ix2010 col: R. Elkins Ex: *C. juglandicola*					
J0069c	KM973313	J0001f	0 /0.99/0	1/0/0		0/0.01/0.99
J0069d	KM973314	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0069e	KM973315	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0069f	KM973316	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0069g	KM973317	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0069h	KM973318	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0069i	KM973319	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0069j	KM973320	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0069k	KM973321	J0069k	0/1/0	1/0/0		0/0.01/0.99
J0069l	KM973322	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0069m	KM973323	J0001e	0.09/0.9/0.01	0.99/0.01/0		0/0.01/0.99
J0070	United States: California, (122° 53' W; 39° 10' N) 13ix2010 col: J.C. Andersen, M.A. Labbé, and R. Elkins Ex: *C. juglandicola*					
J0070a	KM973324	J0051a	0/1/0	1/0/0		0/0.01/0.99
J0070b	KM973325	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0071	United States: California, (122° 55' W; 39° 9' N) 13ix2010 col: J.C. Andersen, M.A. Labbé, and R. Elkins Ex: *C. juglandicola*					
J0071a	KM973326	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0072	United States: California, (122° 54' W; 39° 10' N) 13ix2010 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0072a	KM973327	J0001e	0/0.99/0.01	1/0/0		0/0.01/0.99
J0073	United States: California, (122° 53' W; 38° 59' N) 13ix2010 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0073a	KM973328	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0073b	KM973329	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0074	United States: California, (122° 54' W; 39° 10' N) 13ix2010 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0074a	KM973330	J0001e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0120	France: Parnac (1° 18' E; 44° 29' N) 2vi2011 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0120a	KM973331	J0120a	0/0/0.99		0/0.01/0.99	0.99/0.01/0
J0120b	KM973332	J0120b	0/0.01/0.99		0/0.01/0.99	0.99/0.01/0
J0120c	KM973333	J0120a	0.01/0.02/0.97		0/0.02/0.98	0.96/0.04/0
J0120d	KM973334	J0024a	0/0.01/0.99		0/0.01/0.99	0.99/0.01/0
J0120e	KM973335	J0024a	0/0.01/0.99		0/0.01/0.99	0.98/0.02/0
J0120f	KM973336	J0024a	0/0.01/0.99		0/0.01/0.99	0.98/0.02/0
J0120g	KM973337	J0120a	0/0.01/0.98		0/0.01/0.99	0.97/0.03/0
J0122	France: Aiguille (0° 20' E; 44° 16' N) 2vi2010 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0122a	KM973338	J0024a	0/0.01/0.99		0/0.01/0.99	1/0.01/0
J0122b	KM973339	J0024a	0/0.01/0.98		0/0.01/0.99	0.97/0.03/0
J0122c	KM973340	J0122c	0.01/0.04/0.95		0/0.01/0.99	0.95/0.05/0
J0122d	KM973341	J0024a	0/0/0.99		0/0.01/0.99	0.99/0.01/0
J0122e	KM973342	J0122c	0/0.04/0.96		0/0.01/0.99	0.93/0.07/0
J0122f	KM973343	J0024a	0.02/0.01/0.97		0/0.02/0.98	0.99/0.01/0
J0123	France: Nicole (0° 20' E; 44° 18' N) 2vi2010 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0123a	KM973344	J0024a	0/0.01/0.99		0/0.01/0.99	0.98/0.02/0
J0124	France: Bordeaux (0° 17.227' E; 44° 34.467' N) 3vi2010 col: J.C. Andersen and M.A. Labbé Ex: *C.*					

	juglandicola					
J0124a	KM973345	J0124a	0/0.01/0.99		0/0.01/0.99	0.99/0.01/0
J0125	France: Bordeaux (0° 17.227' E; 44° 34.467' N) 3vi2010 col: J.C. Andersen and M.A. Labbé Ex: *M. coryli*					
J0125A	KM973346	J0027b	0.93/0.05/0.02	0/0.08/0.92	0.98/0.02/0	
J0137	Italy: Verolengo (8° 1' E; 45° 11' N) 22vi2010 col: J.C. Andersen and M.A. Labbé Ex: *M. coryli*					
J0137A	KM973347	J0023a	0.02/0.04/0.94		0/0.05/0.95	0.76/0.24/0
J0138	Italy: Verolengo (7° 57' E; 45° 10' N) 22vi2010 col: J.C. Andersen and M.A. Labbé Ex: *M. coryli*					
J0138A	KM973348	J0019a	0.07/0.01/0.92		0/0.35/0.66	0.99/0.01/0
J0138B	KM973349	J0138b	0.01/0/0.99		0/0.01/0.99	0.99/0.01/0
J0143	Italy: Verolengo (7° 57' E; 45° 10' N) 22vi2010 J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0143A	KM973351	J0138b	0/0.01/0.99		0/0.01/0.99	0.99/0.01/0
J0151	United States: California, (121° 59.39' W; 38° 30.912' N) 12vii2011 col: J.C. Andersen Ex: *Chromaphis juglandicola*					
J0151A	KM973352	J0001e	0/1/0	0.99/0.01/0		0/0.01/0.99
J0151B	KM973353	J0001e	0/0.99/0.01	0.99/0.01/0		0/0.01/0.99
J0152	United States: California, (122° 4.307' W; 38° 57.938' N) 12vii2011 col: J.C. Andersen Ex: *C. juglandicola*					
J0152a	KM973354	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0152B	KM973355	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0152C	KM973356	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0153	United States: California, (122° 1.931' W; 39° 14.835' N) 12vii2011 col: J.C. Andersen Ex: *C. juglandicola*					
J0153A	KM973357	J0001e	0/0.99/0.01	1/0/0		0/0.01/0.99
J0153B	KM973358	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0155	United States: California, (121° 41.405' W; 39° 16.544' N) 12vii2011 col: J.C. Andersen Ex: *C. juglandicola*					
J0155B	KM973359	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0156	United States: California, (121° 35.361' W; 39° 6.771' N) 12vii2011 col: J.C. Andersen Ex: *C. juglandicola*					
J0156A	KM973360	J0001e	0.006/0.992/0.002	0.99/0.01/0		0/0.01/0.99
J0157	United States: California, (121° 35.361' W; 38° 52.716' N) 12vii2011 col: J.C. Andersen Ex: *C. juglandicola*					
J0157A	KM973361	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0157B	KM973362	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0161	Iran: Esfahan 15v2011 col: E. Nader Ex: *C. juglandicola*					
J0161E	KM973363	J0161e	0/1/0	1/0/0		0/0.01/0.99
J0161F	KM973364	J0161f	0.01/0.68/0.31			0/0.05/0.95
J0163	Iran: Tehran 24iii2004 col: E. Nader Ex: *C. juglandicola*					
J0163A	KM973365	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0163B	KM973366	J0001f	0/0.99/0.01	0.99/0.01/0		0/0.01/0.99
J0163C	KM973367	J0001f	0.03/0.95/0.02	0.99/0.01/0		0/0.01/0.99
J0163D	KM973368	J0001e	0/0.86/0.14	0.99/0.01/0		0/0.09/0.91
J0163E	KM973369	J0001f	0.02/0.98/0	0.99/0.01/0		0/0.01/0.99
J0163F	KM973370	J0001f	0.04/0.94/0.02	0.99/0.01/0		0/0.01/0.99
J0163G	KM973371	J0001f	0/0.92/0.08	1/0/0		0/0.01/0.99
J0163H	KR074103	J0001f	0/0.99/0.01	1/0/0		0/0.01/0.99
J0163I	KR074104	J0001f	0/0.99/0.01	1/0/0		0/0.01/0.99
J0163J	KR074105	J0163J	0/1/0	1/0/0		0/0.01/0.99
J0173	United States: California, (39.11296, -121.63568) 27ix2011 col: J.C. Andersen Ex: *Panaphis*					

	juglandis					
J0173A	KM973372	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0173B	KM973373	J0001e	0/0.99/0.01	1/0/0		0/0.01/0.99
J0174	United States: California, (39.276, -121.69147) 27ix2011 col: J.C. Andersen Ex: *P. juglandis*					
J0174B	KM973374	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0174C	KM973375	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0174D	KM973376	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0178	United States: California, (39.11296, -121.63568) 27ix2011 col: J.C. Andersen Ex: *P. juglandis*					
J0178B	KM973377	J0178b	0/1/0	1/0/0		0/0.01/0.99
J0178C	KM973378	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179	United States: California, Escalon (37.754141, -120.923234) 5vi2012 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0179A	KM973379	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179B	KM973380	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179C	KM973381	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179D	KM973382	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179E	KM973383	J0179e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0179F	KM973384	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179G	KM973385	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179H	KM973386	J0001e	0.02/0.96/0.02	0.99/0.01/0		0/0.01/0.99
J0179I	KM973387	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0179J	KM973388	J0001e	0/0.99/0	1/0/0		0/0.01/0.99
J0179K	KM973389	J0179k	0/0.99/0.01	1/0/0		0/0.01/0.99
J0179L	KM973390	J0001e	0/0.99/0	0.99/0.01/0		0/0.01/0.99
J0188	United States: California, Newark (37.561215, -122.041887) 30viii2012 col: J.C. Andersen and M.A. Labbé Ex: *C. juglandicola*					
J0188A	KM973391	J0001e	0.02/0.97/0.01	1/0.01/0		0/0.01/0.99
J0188B	KM973392	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0188C	KM973393	J0001e	0/1/0	0.99/0.01/0		0/0.01/0.99
J0188D	KM973394	J0001e	0.01/0.99/0	0.99/0.01/0		0/0.01/0.99
J0188E	KM973395	J0001f	0/1/0	1/0/0		0/0.01/0.99
J0188F	KM973396	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0188G	KM973397	J0001e	0.05/0.94/0.01	0.99/0.01/0		0/0.01/0.99
J0188H	KM973398	J0001e	0/1/0	1/0/0		0/0.01/0.99
J0188I	KM973399	J0001e	0/1/0	0.99/0.01/0		0/0.01/0.99
J0188J	KM973400	J0001e	0.05/0.94/0.01	0.99/0.01/0		0/0.01/0.99
J0189	Italy: col: J.C. Andersen and M.A. Labbé Ex: *Myzocallis coryli*					
J0189A	KM973401	J0189a	0.02/0.01/0.97		0/0.14/0.86	0.98/0.02/0

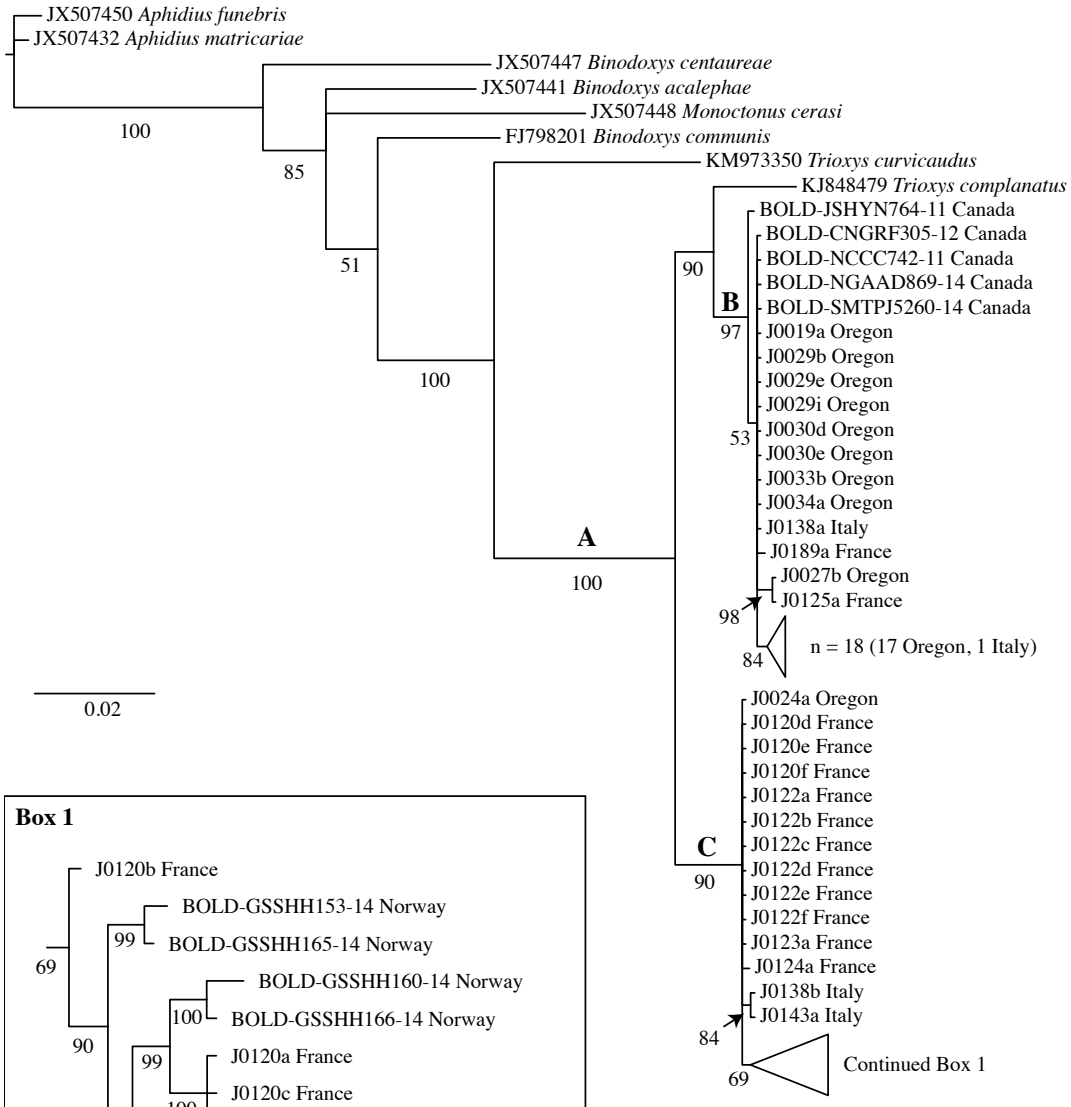

Figure 4.1 – Bayesian majority rule consensus tree of relationships between individuals identified as *T. pallidus* and published sequences. Branch lengths are drawn proportional to the number of substitutions per site, with a scale shown in the bottom left corner and Bayesian posterior probabilities (BPP) are shown below each branch, except when noted. One clade ("A") includes all sequences for *T. pallidus* generated in this study. A second clade ("B") includes only individuals reared from *M. coryli* or from unknown sources, and a third clade ("C") includes individuals from tree-feeding aphids in the tribe Panaphidini in Europe, Iran, and North America.

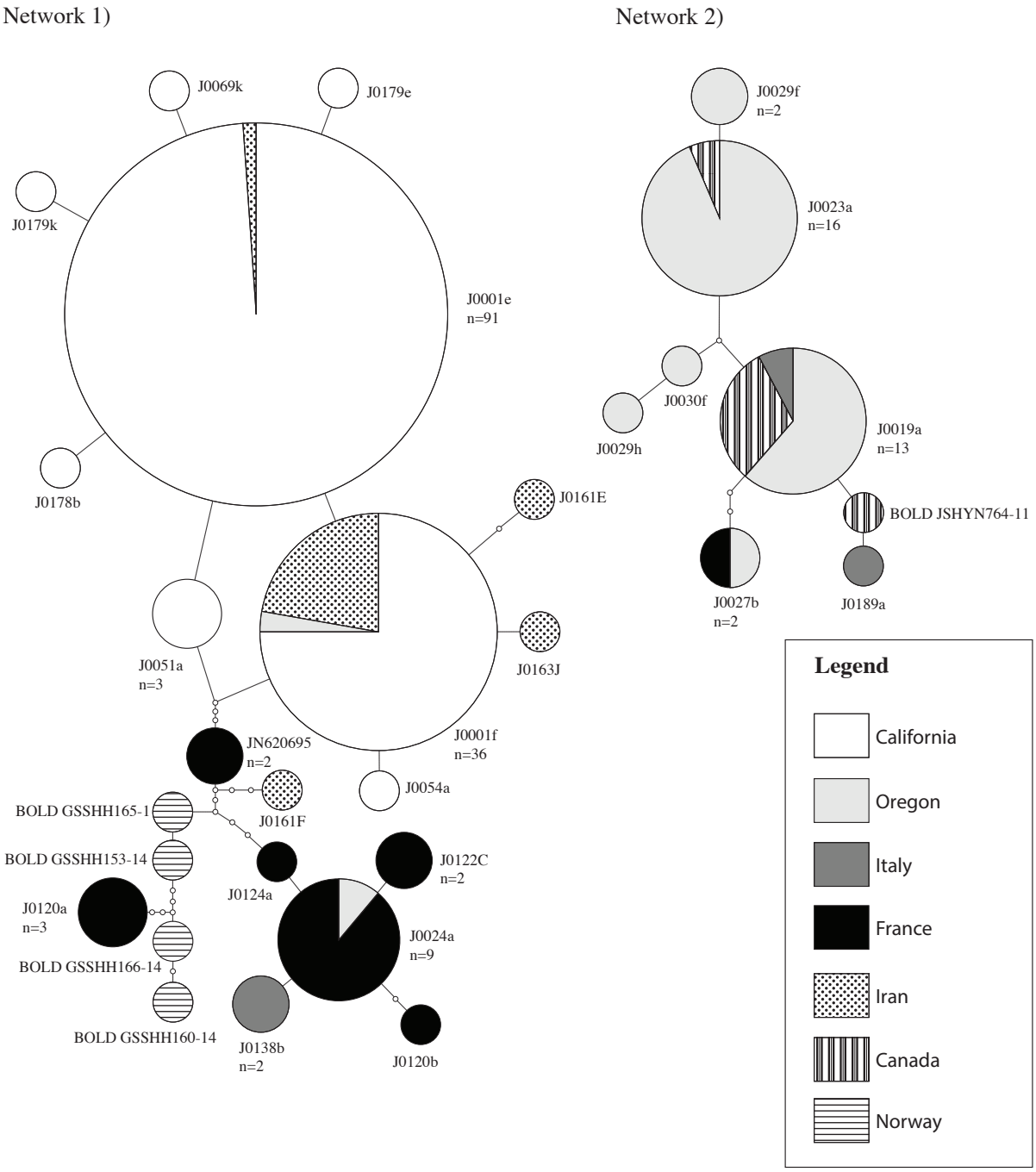

Figure 4.2 – TCS network diagram: geographic associations. Haplotype names and the number of individuals belonging to each haplotype are drawn adjacent to a circle representing each haplotype, and the size of the circle is proportional to the number of sequences matching that haplotype. Circles are colored to represent the geographic location from which an individual was collected, and branches with open circles are drawn to represent the number of base pair differences between each haplotype.

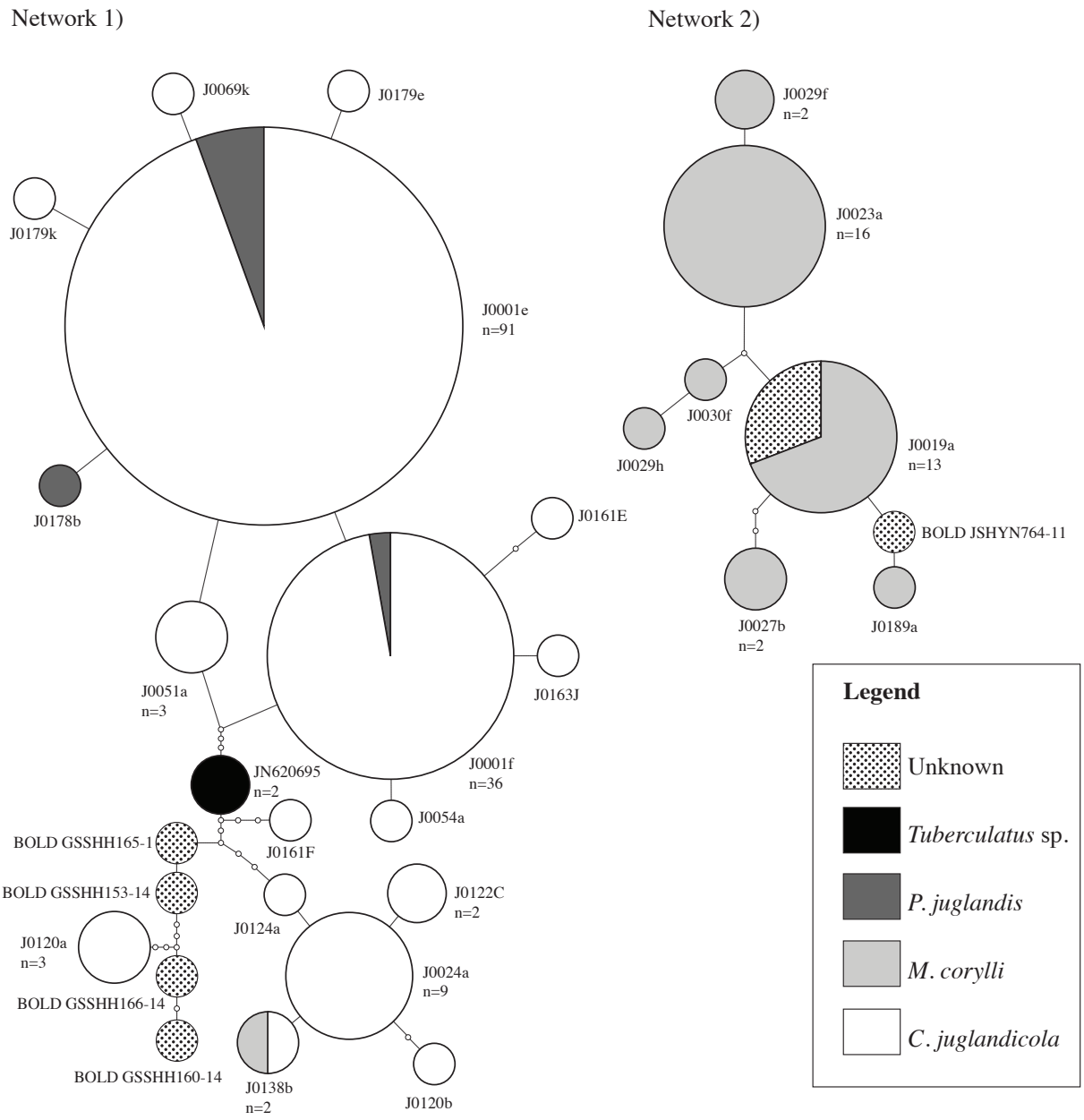

Figure 4.3 – TCS network diagram: host associations. Drawn as per figure 4.2, except that circles are colored to represent host-associations, if known, for aphid species from which each individual emerged.

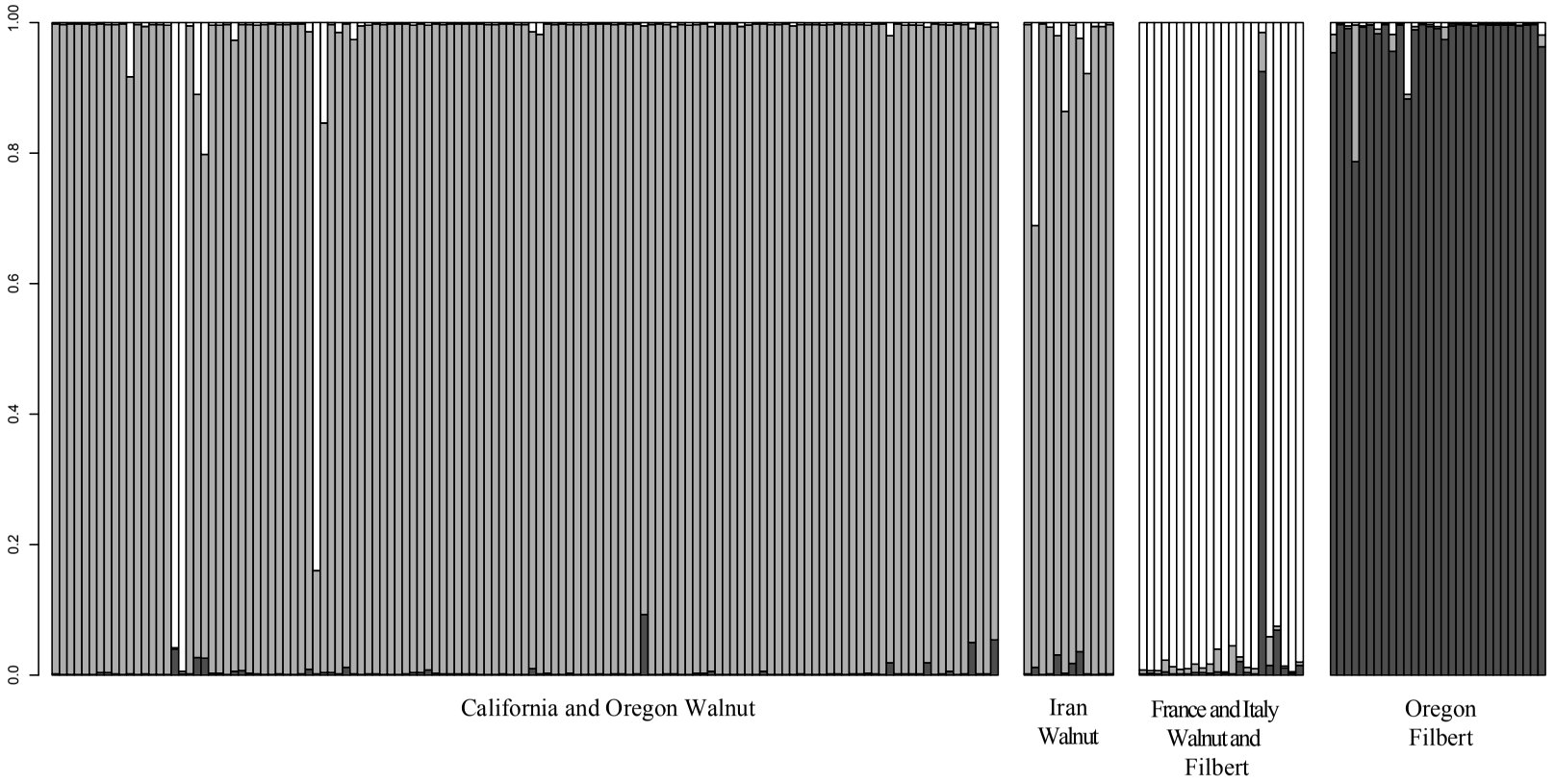

Figure 4.4 – Probability of assignment of individuals to genetic clusters based on the analysis of microsatellite data. Each column represents the probability of assignment of an individual to each of three genetic clusters (colored white, light grey, and dark grey, respectively) using STRUCTURE. Individuals are grouped by the geographic localities and/or aphid hosts from which they were collected.

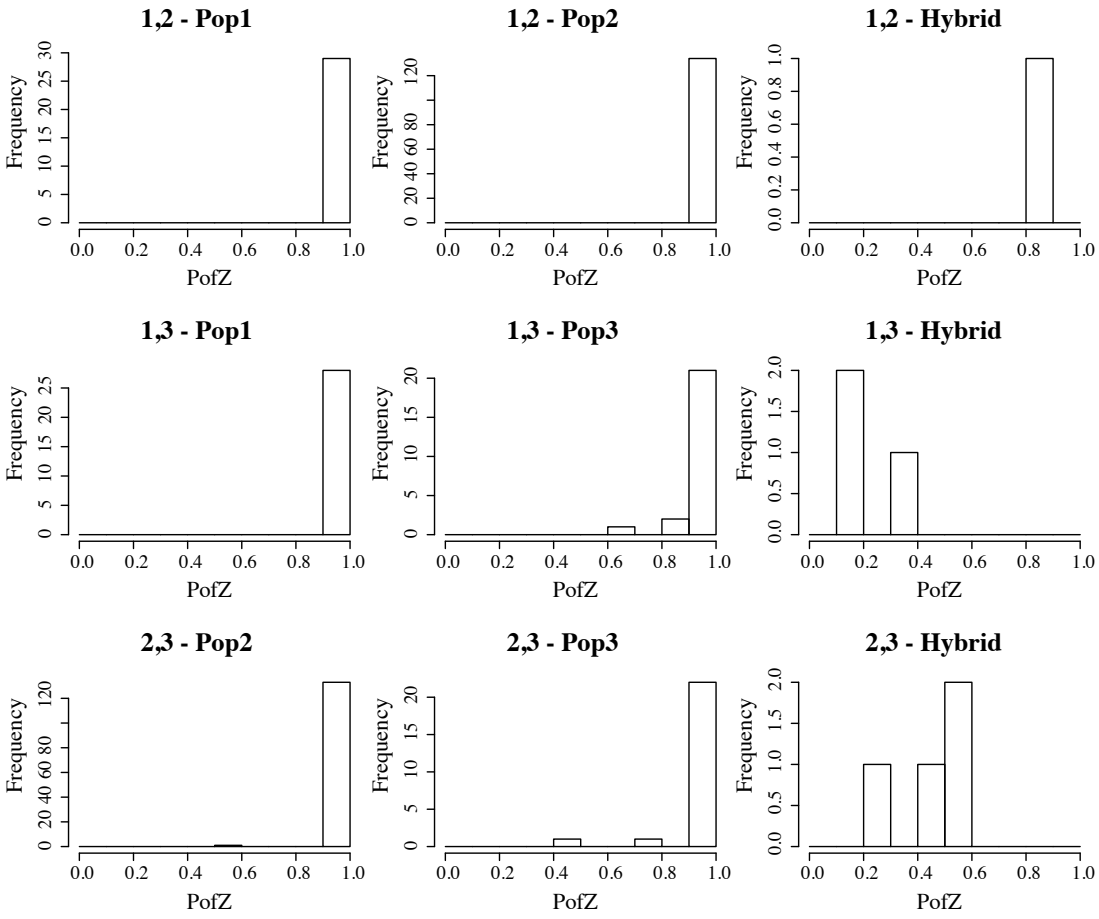

Figure 4.5 – Probability of assignment to pure or hybrid categories using NewHybrids. Each row shows the probability of assignment of individuals to either one of the pure or hybrid classes for each pairwise analysis using NewHybrids. Hybrid scores were calculated by summing the probabilities for all of the hybrid classes.

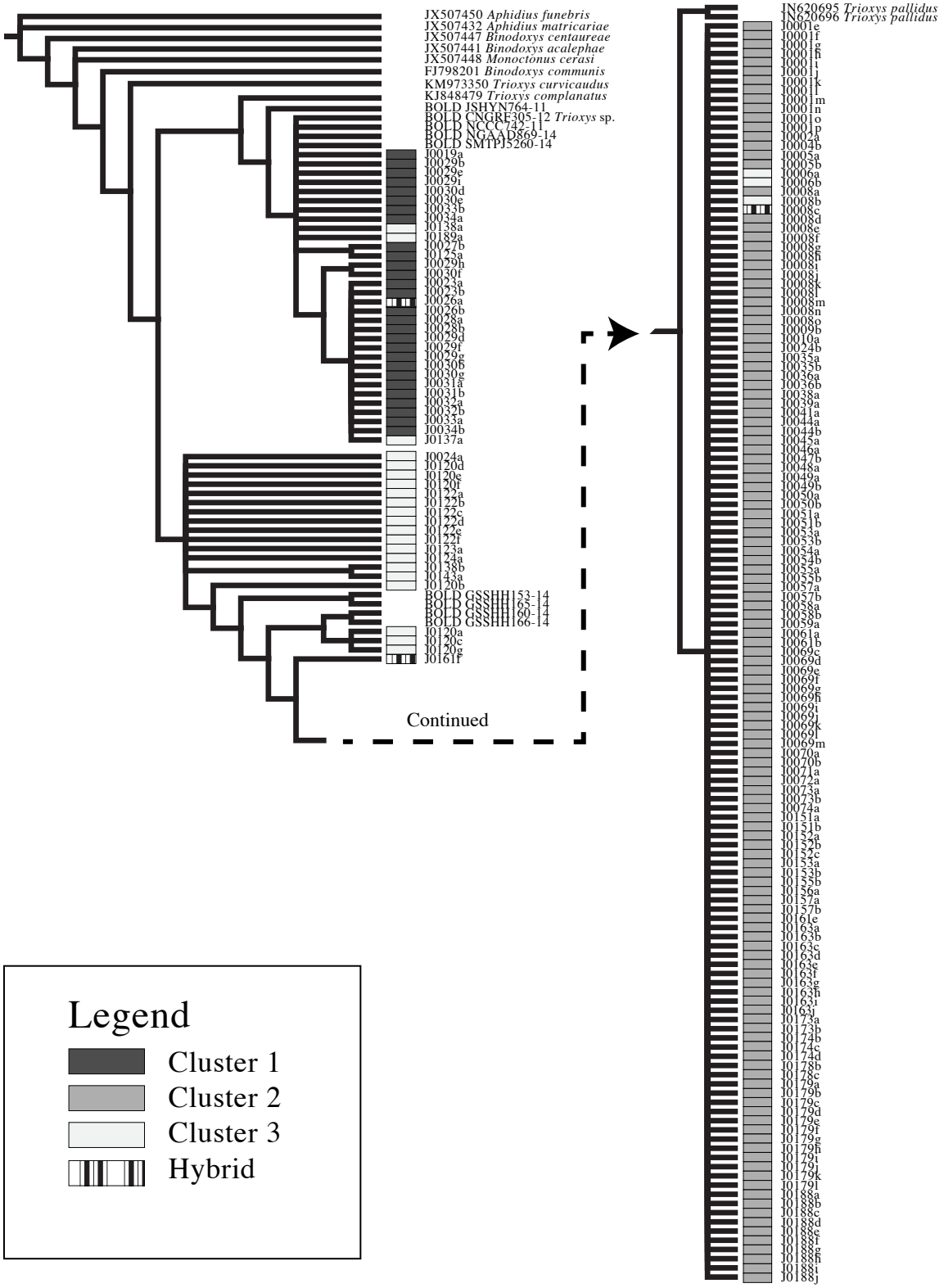

Figure 4.6 – Comparison of results from mtDNA and nuDNA analyses. For *T. pallidus* individuals, adjacent to each ID number the genetic cluster to which each individual was assigned using STRUCTURE is drawn (Q > 0.80 for Clusters 1 – 3; Q < 0.8 for Hybrid).

CHAPTER 5: POST-RELEASE GENETIC SURVEYS UNCOVER A GEOGRAPHIC MOSAIC OF COEVOLUTION IN THE WALNUT APHID BIOLOGICAL CONTROL PROGRAM.

Abstract

The geographic mosaic theory of coevolution predicts that coevolutionary interactions are both geographically and temporally dynamic, and that reciprocal coevolution between interacting species occurs in some locations (coevolutionary hotspots), but not in others (coevolutionary coldspots). Whether geographic mosaics of coevolution occur in the context of classical biological control is unknown, but if it does, then localized breakdowns in biological control services might be expected to develop as populations oscillate between being coevolutionary hotspots and coldspots. We surveyed orchards for walnut aphid, *Chromaphis juglandicola,* and its parasitoid *Trioxys pallidus* and using population genomics we found positive evidence for the presence of a geographic mosaic of coevolution in the interaction between these two species. We also found that a greater number of the aphid populations exhibited significant levels of population differentiation than did *T. pallidus* populations. We subsequently discuss the implications of a geographic mosaic of coevolution and of potentially differing rates of evolution between parasitoids and their target pests for the sustainability of pest control services.

Introduction

One of the most important challenges in evolutionary biology has been to identify the factors and processes that have shaped the diversity of species observed on our planet (Darwin 1859; Haldane 1947; Pennisi 2005). This diversity has resulted, in part, from interactions among species within communities through the process of coevolution (Hembry *et al*. 2014), the reciprocal evolutionary change of interacting species (Ehrlich & Raven 1964; Janzen 1980; Thompson 2014). Coevolution is thought to have played an important role in the diversification of plants and their pollinators (Ehrlich & Raven 1964) and herbivores (Bernays & Graham 1988), and has been particularly well studied between aphids and their parasitoids (e.g. Dion *et al*. 2011; Henter & Via 1995; Nyabuga *et al*. 2012; Rouchet & Vorburger 2012; Schmid *et al*. 2012) where defensive endosymbionts also play an important role (Bilodeau *et al*. 2013; Oliver *et al*. 2003; Rouchet & Vorburger 2014; Vorburger 2014).

While coevolutionary interactions, by definition, exist at the nexus of ecological and evolutionary time scales and theories (Torres 2009), it has also been proposed that they can occur at a much broader landscape scale through the geographic mosaic theory of coevolution (Thompson 1994, 2005). From this theory, the spatial structure of populations and the geneflow between them is expected to be an important part of the coevolutionary process (Gibert *et al.* 2013). Thus, coevolution is expected to be both spatially and temporally dynamic, with populations of interacting organisms oscillating between periods of reciprocal coevolution (termed coevolutionary hotspots) and periods where selection may be acting upon one or neither of the species in the pair (termed coevolutionary coldspots). Given its potential importance for understanding patterns of coevolution, it is perhaps not surprising that geographic mosaics have been observed for a

variety of organisms and interactions including plants and herbivores (Siepielski & Benkman 2005; Muola et al. 2010; Vermeer *et al.* 2012), plants and pollinators (Thompson & Cunningham 2002; Thompson & Fernandez 2006), predators and prey (Brodie & Ridenhour 2002), and hosts and parasites (Dixon *et al.* 2009; Thompson 2009; Lorenzi & Thompson 2011; Vergara *et al.* 2013).

The geographic mosaic theory of coevolution is also thought to be applicable to managed agricultural ecosystems (Bousset & Chevre 2013). In these systems metapopulation dynamics (Ives & Settle 1997) and coevolutionary interactions (Holt & Hochberg 1997) have long been thought to play an important role in influencing the biological control services provided by natural enemies in the suppression of pest abundance (Hoddle 2004; Van Driesche *et al*. 2010). Biological control programs are known to be well suited for the study of evolution (Roderick & Navajas 2003; Roderick *et al*. 2012); however, whether these programs are affected by spatial and temporal variation in coevolutionary interactions, as predicted by the geographic mosaic theory of coevolution, is unknown. This lack of knowledge may, in part, be explained by a predominant focus on pre-introduction surveys for natural enemies without sufficient emphasis on longer-term post-introduction monitoring (Mills 2000; McCoy & Frank 2010). It may also be explained by the fact that detection of geographic mosaics of coevolution has relied upon the *a priori* identification and measurement of adaptations among the interacting species, such as proboscis length for pollinators and corolla length for flowers (Pauw *et al*. 2009) or rostrum length for herbivores and pericarp thickness for fruit (Toju & Sota 2006). Adaptations among interacting species in agricultural systems may be more difficult to detect due to the high level of disturbance in these systems, and the selective breeding of crop plants for specific traits. In addition, in host-parasitoid interactions – a common interaction in biological control programs – numerous factors influence parasitism rates including genotype x genotype interactions, the presence of defensive endosymbionts, the type of host plant, and/or host color morphs (Bilodeau *et al*. 2013). Consequently, molecular techniques may be better suited for the identification of geographic mosaics of coevolution in these environments. Several studies have used molecular methods to gain further insights into previously identified geographic mosaics of coevolution (e.g. Dupas *et al*. 2009; Parchman *et al*. 2011; Severine *et al*. 2013). Only recently, however, has a population genomics approach been proposed for identification of potential geographic mosaics of coevolution (Vermeer *et al*. 2011). The advantages of this approach are that it can be implemented in the absence of *a priori* knowledge of adaptive traits (Vermeer *et al*. 2011) and addresses previous concerns about appropriate methods for testing the geographic mosaic theory of coevolution (Gomulkiewicz *et al*. 2007).

If, as expected, coevolutionary interactions are important for biological control services and the geographic mosaic theory of coevolution predicts these interactions to be spatially and temporally dynamic, then localized breakdowns in biological control services might be expected as populations transition from being coevolutionary hotspots to coldspots (or vice versa). Such breakdowns have recently been observed in the walnut aphid biological control program in California where localized increases in abundance of walnut aphid, *Chromaphis juglandicola* (Kaltenbach) (Hemiptera: Aphididae), has led to

a resumption of in-season insecticidal treatments (Hougardy & Mills 2008). Walnuts are one of the principle tree crops in the state of California both in terms of annual value (~$1.8 billion USD) and acreage of agricultural landscape occupied (>113,000 ha) (USDA 2014). Walnut aphid has been present in California for nearly as long as commercial walnut orchards have exhisted (Davidson 1914). They are active from March until early December (Sluss 1967), and cause reductions in the quality and size of the nuts (Michelbacher & Ortega 1958). To control walnut aphid, the parasitoid wasp *Trioxys pallidus* (Haliday) (Hymenoptera: Braconidae) was introduced to California from southern France, which resulted in establishment in the southern and coastal regions, but failed to establish the parasitoid in the primary walnut growing region of the Central Valley (Schlinger *et al.* 1960). Subsequently, a second introduction from Iran led to widespread establishment and reduction of walnut aphid densities throughout California (van den Bosch *et al.* 1979).

In a recent study we suspected – incorrectly – that hybridization between populations descended from the separate introductions of *T. pallidus* may have played a role in the observed breakdowns (Andersen & Mills in prep). While conducting genetic surveys of *T. pallidus* for that study, we found evidence of geographically-based population structure in the different walnut growing regions of California (JCA and NJM unpublished data). This genetic variation in populations of *T. pallidus*, coupled with the observation of localized breakdowns, suggested that this aphid-paraitoid interaction may be influenced by a geographic mosaic of coevolution. Therefore, the obejctives of this study were 1) to examine whether we could use the population genomics approach presented by Vermeer *et al.* (2011) to detect the presence of a geographic mosaic of coevolution in the walnut aphid biological control program, and 2) to compare levels of genetic differentiation between populations of *T. pallidus* and *C. juglandicola* and comment on the effects of differing rates of evolution for the stability of biological control services.

Methods

Sampling Locations

To apply the population genomic approach of Vermeer *et al*. (2011) to investigate the interaction of *C. juglandicola* and *T. pallidus* in walnut orchards, sampling was conducted in both California and France between 2010 and 2014. Two orchards with only one of the two interacting species present were needed as reference coevolutionary coldspot locations. The reference coldspot for *T. pallidus* was located near Yuba City, CA where we found *T. pallidus* parasitizing an alternative host, the dusky-veined aphid (*Panaphis juglandis* [Goeze] [Hemiptera: Aphididae]). The reference coldspot for *C. juglandicola* was located near Linden, CA where we collected aphids, but adults of *T. pallidus* and aphid mummies were absent. We sampled five additional orchards to test both the aphids and parasitoids present for evidence of population structure and variation in genetic diversity that could be consistent with a geographic mosaic of coevolution. Full details of each of the sampling locations are presented in Table 5.1.

At each sampling location we collected individuals identified as *T. pallidus* either by aspirating adults or by collecting mummified walnut aphids and placing small cut-out

sections of leaf material with each mummy in a glass vial (9.5 mm x 3 mm). These vials were closed with a foam stopper, and stored at room temperature until adults emerged. Whether aspirated, or reared, adults of *T. pallidus* were then stored in 95% ethanol at -20°C for molecular analysis. Individuals of *C. juglandicola* were collected in the field and immediately placed in 95% ethanol and then stored at -20°C for molecular analysis. Effort was taken to collect only a few individuals per tree and to prioritize sampling from as many different trees as possible in each orchard to reduce the sampling of clonally related individuals (Lozier *et al*. 2007).

DNA Extraction and Microsatellite Genotyping

DNA was extracted from adult females of *T. pallidus* and *C. juglandicola* by grinding individuals with a mortar and pestle and then using the modified DNA extraction protocols presented in Andersen & Mills (2014, in prep). Standard PCR protocols were then used to amplify 15 polymorphic microsatellite markers for *T. pallidus* and 12 polymorphic microsatellite markers for *C. juglandicola* following protocols presented in Andersen & Mills (2014). Briefly, microsatellite loci were amplified from 7 to 20 aphids and/or parasitoid females from each location using fluorescently labeled primers, and PCR products for up to four loci were pooled before genotyping so that no two loci with the same fluorescent label were combined. Products were then genotyped on an Applied Biosystems 3730XL DNA Analyzer at the University of California Berkeley DNA Sequencing Facility using the LIZ 600 size standard, and fragment lengths were then scored using the Microsatellite Plug-in for Geneious Pro v. 5.6.2 (Drummond *et al*. 2012).

Population Genomic Analyses

To test for the presence of a geographic mosaic of coevolution, we followed the approach presented by Vermeer *et al*. (2011). Briefly, this approach addresses the three components of the geographic mosaic theory of coevolution by identifying; 1) coevolutionary hot- and coldspots, 2) selection mosaics, and 3) trait-remixing (Gomulkiewicz *et al*. 2007; Thompson 1994, 2005). Neutral genetic loci for two (or more) interacting species are used to survey individuals at a number of geographic locations where both species co-occur as well as a single location for each species where its counterpart is absent. Using the latter as known coevolutionary coldspots, pairwise comparisons of genetic diversity (e.g. F_{ST}, G_{ST}, Rho_{ST}, etc.) between these and other locations can be estimated for each locus and used to look for outlier loci. If outliers are detected at a particular location for both interacting species, the location may be a coevolutionary hotspot, whereas if outliers are present for only one or neither of the interacting species, the location may be a coevolutionary coldspot (i.e. components 1 and 2 of the theory). These same neutral loci can then be used to estimate levels of geneflow between locations (i.e. component 3 of the theory). Finally, evidence for population structure (genetic diversity) and outlier loci can be used to identify potential hot and coldspots locations, which in conjunction with measurements of phenotypic or behavioral traits, can be used to confirm whether the interacting species are under reciprocal selection.

To implement this approach we used the software package GenePop (Raymond & Rousset 1995; Rousset 2008) to calculate summary statistics for each population including; the number of observed and expected heterozygotes (*Ho* and *He*, respectively), departures from Hardy-Weinberg equilibrium (HWE), and the presence of locus-by-locus linkage disequilibrium (LD). We then used GenePop to estimate measures of population differentiation based on both F_{ST} and Rho_{ST} as the latter incorporates fragment sizes and may be more appropriate for microsatellite loci (Hardy *et al.* 2003; Slatkin 1995), while the former is independent of allele size and maybe more approriate in the face of scoring errors (Donnelly & Townson 2000; Zheng *et al.* 1996) or deviations from the simple-stepwise-mutation model (Putman & Carbone 2014). We then estimated recent migration rates (i.e. the proportion of individuals in a population that were estimated to be derived from a second population) between each population using the BayesAss Edition 3.0 (BA3) software package (Wilson & Rannala 2003). Analyses were conducted using maximal mixing parameters (0.99) for allele frequencies, migration rates, and inbreeding coefficiencies, and a runtime of 10 million generations with a burnin period of 1 million generations. Results were then visualized using the program Tracer (Rambaut & Drummond 2007), and for all *T. pallidus* populations, burnin periods were adjusted to 1.5 million generations to ensure that MCMC runs reached stationarity.

Detection of Outlier Loci

We then estimated locus-specific measures of genetic differentiation for both *C. juglandicola* and *T. pallidus* using both F_{ST} and Rho_{ST} in GenePop (Raymond & Rousset 1995; Rousset 2008) based on pairwise comparisons between the reference coldspots and each candidate location. The "boxplot" function in the statistical software package R v 3.1.3 (R Core Team 2015) was used to examine the distribution of the locus-specific estimates for each location and outlier loci were detected by falling outside of the whiskers representing 1.5 times the interquartile range.

Results

Population Genomic Analyses

Both the observed heterozygosities (mean *Ho* of 0.306 for *T. pallidus* and 0.139 for *C. juglandicola*) and expected heterozygosities (mean *He* of 0.381 for *T. pallidus* and 0.175 for *C. juglandicola*) appeared low. Two populations for *T. pallidus* (Arbuckle, CA and Parnac, France) displayed significant deviations from HWE ($X^2 = 54.25$, df = 26, $P < 0.001$, and $X^2 = 66.70$, df = 28, $P < 0.001$ for the two *T. pallidus* populations, respectively), as did one population for *C. juglandicola* (Arbuckle, CA; $X^2 = \infty$, df = 20, $P < 0.001$). For all three populations these differences were still significant after Bonferonni correction for multiple comparisons.

Two of the 105 pair-wise LD comparisons for the microsatellite loci amplified from *T. pallidus* (*Tp*_MSAT8 and *Tp*_MSAT12 [$X^2 = 21.35$, df = 12, $P = 0.045$]; *Tp*_MSAT13 and *Tp*_MSAT17 [$X^2 = 22.80$, df = 12, $P = 0.030$]), and three of the 66 pair-wise LD comparisons for the microsatellite loci amplified from *C. juglandicola* (*Cj*_MSAT5 and *Cj*_MSAT9 [$X^2 = 15.05$, df = 6, $P = 0.019$]; *Cj*_MSAT5 and *Cj*_MSAT19 [$X^2 = 13.11$, df = 6, $P = 0.041$]; and *Cj*_MSAT8 and *Cj*_MSAT19 [$X^2 =$

14.62, df = 2, $P < 0.001$]) showed a significant presence of LD. However, only the pairwise comparison between *Cj*_MSAT8 and *Cj*_MSAT19 retained significant LD after Bonferonni correction for multiple comparisons.

Measures of population differentiation (F_{ST} and Rho_{ST}) were significant for both *T. pallidus* and *C. juglandicola* populations (Table 5.2). For *T. pallidus,* the Modesto, CA and Parnac, France populations showed significant differentiation from all other populations (though only the Parnac population remained significant after Bonferonni correction for multiple comparisons). In contrast, for *C. juglandicola* the Arbuckle and Upper Lake, CA and the Parnac, France populations all showed significant differentiation from all other populations (and all remained significant after Bonferonni correction for multiple comparisons). Consistent with the presence of a geographic mosaic of coevolution we also found that geneflow (i.e. migration rates) varied between population pairs for both of the interacting species (Table 5.3).

Detection of Outlier Loci

Preliminary analyses indicated that two loci (*Cj*_MSAT1 and *Cj*_MSAT16) for *C. juglandicola* and one locus (*Tp*_MSAT12) for *T. pallidus* were detected as outliers in all population comparisons. As indicated by Vermeer *et al.* (2011), such loci are likely associated with regions of the genome that are under selection, but because they did not vary geographically across population comparions, these loci do not represent the presence of reciprocal coevolution as predicted by the geographic mosaic theory and thus were excluded. After removal of these three loci, we identified outlier loci in four of the pair-wise comparisons for *T. pallidus* using F_{ST} and three of the pair-wise comparisons using Rho_{ST} (Figure 5.1). For *C. juglandicola*, outlier loci were detected in four pair-wise comparisons using both F_{ST} and Rho_{ST}, though the individual populations where outlier loci were detected varied (i.e. Modesto, CA for F_{ST} and Arbuckle, CA for Rho_{ST}). Outlier loci were detected at two locations (Parnac, France and Newark, CA) for both *T. pallidus* and *C. juglandicola* regardless of whether F_{ST} or Rho_{ST} was used as a summary statistic, and for one additional population (Arbuckle, CA) for F_{ST} as a summary statistic. Based on the population genomics approach for detecting the presence of a geographic mosaic of coevolution we therefore identified the Parnac, France; Newark, CA; and Arbuckle, CA populations as putative coevolutionary hotspots with outlier loci detected for both interacting species, and the Modesto, CA and Upper Lake, CA as putative coevolutionary coldspots due to the detection of outlier loci for only one of the two interacting species (Figure 5.2).

Discussion

The study of coevolutionary interactions has not only helped to explain patterns of species diversity (Bernays & Graham 1988; Ehrlich & Raven 1964; Hembry *et al*. 2014; Janzen 1980; Thompson 2014), but has also contributed to our understanding of best management practices for natural resources (Carroll 2011; Kinkel *et al*. 2011; Rammel *et al*. 2007). Coevolution has also long been thought to play an important role in the sustainability of biological control services (Holt & Hochberg 1997; Kraaijeveld & Godfray 1999; Jones *et al*. 2014), and forms a central component of the enemy release

hypothesis (Liu & Stiling 2006), which is often invoked to explain the increased invasiveness of exotic pests and weeds. However, whether geographic mosaics of coevolution (Thompson 1994, 2005), with oscillations between periods during which reciprocal coevolution is (hotspots) and is not (coldspots) occurring, affect the sustainability of biological control service is unknown. If it does, then these oscillations could likely result in localized breakdowns in biological control services, much like the patterns observed more recently in the walnut aphid biological control program (Hougardy & Mills 2008). In this context, then perhaps our finding of a geographic mosaic of coevolution in the interaction between *C. juglandicola* and *T. pallidus* may help to explain why some growers have resorted to in-season insecticidal applications while others have not.

While geographic mosaics of coevolution have been observed in numerous study systems, two explanations for why this may be the first study to observe its presence in a biological control interaction set in an agricultural crop are: 1) that too few longer-term post-release studies have been undertaken (Mills 2000; McCoy & Frank 2010), and 2) that it may be difficult to identify *a priori* which adaptive traits to measure. The latter is of particular concern for aphid-parasitoid interactions where numerous factors can influence host susceptability to parasitism including the presence of defensive symbionts, aphid and parasitoid genotype x genotype interactions, as well as aphid color morphs (Bilodeau *et al.* 2013; Vorburger 2014). Previous studies have suspected the presence of geographic mosaics of coevolution as a potential explanation for the invasiveness of knapweeds (*Centaurea maculosa* Lamarck, and *C. diffusa* Lamarck [Asterales: Asteraceae]) in North America, but this could not be confirmed (Callaway *et al*. 2005). Therefore, we suspect that if the population genomics approach suggested by Vermeer *et al*. (2011) were to be applied more broadly to biological control interactions in managed ecosystems, additional geographic mosaics of coevolution would undoubtedly be observed.

In a pioneering paper, Holt and Hochberg (1997) outlined a range of factors (e.g. metapopulations dynamics, temporal variability in selective pressures, coevolutionary interactions) that might explain why the evolution of resistance is so rare in biological control programs. However, resistance is either known, or is suspected, to have occurred in at least some biological control programs (e.g. Ives & Muldrew 1984; Goldson *et al*. 2014). For the larch sawfly in Canada resistance to parasitism may have arisen due to the accidental importation of resistant host strains (Ives & Muldrew 1984), while for Argentine stem weevil in New Zealand parasitism rates have decreased nearly 50% in the last 10-15 years (Goldson *et al*. 2014). In the latter case, coevolutionary interactions would be expected to be weak due to the use of asexual biological control agents and the homogeneity of the New Zealand rangeland ecosystem. Thus the longer-term evolutionary outcome of biological control programs may represent a continuum from sustained effective control when coevolutionary interactions are strong, to breakdown and failure when they are weak. Geographic mosaics with reciprocal selection and trait remixing would occupy an intermediate position along the continuum and be characterized by temporary breakdowns in effective control at a localized scale, and yet

sustained control at a regional or landscape scale due to connectivity and movement between local populations.

An additional finding from this study was that the measure of genetic differentiation employed (F_{ST} or Rho_{ST}) influenced the number of locations identified as either coevolutionary hot- or coldspots. Two locations (Parnac, France and Newark, CA) were identified as coevolutionary hotspots during outlier tests using both statistics. However, for another location (Arbuckle, CA) outlier loci were detected for *C. juglandicola* using both statistics, but were detected for *T. pallidus* using F_{ST} only and not when using Rho_{ST}. Both test statistics are expected to be equivalent in populations with high migration rates and that have recently diverged (Hardy *et al*. 2003; Slatkin 1995; Rousset 1996). While tests exist to determine which statistic may be more appropriate for a given dataset (Hardy *et al*. 2003; Hardy & Vekemans 2002), it is advised to report both (Heller & Siegismund 2009; Putman *et al*. 2014), as we have done here. An additional study that measures morphological and behavioral trait variation at each of our study sites will be required to verify the presence of coevolutionary hot- and coldspots, and to verify which statistic is more appropriate for this system.

Population Structure of T. pallidus *and* C. juglandicola

Both *C. juglandicola* and *T. pallidus* showed evidence of population differentiation in this study, but the level of differentiation was lower for the parasitoid than for the aphid (Table 5.2). For *T. pallidus*, two populations showed significant differentiation (Modesto, CA and Parnac, France; though only the Parnac, France was significant after applying a Bonferonni correction), whereas for *C. juglandicola* three populations showed significant differentiation (Arbuckle, CA; Upper Lake, CA; and Parnac, France; all were significant after applying a Bonferonni correction). It is unclear whether the observed difference in levels of population differentiation was due to the amount of time each species has been present in western North America (>100 years for *C. juglandicola* versus 47 years for *T. pallidus* [Davidson 1914; van den Bosch *et al*. 1979]) or due to different rates of evolution.

Some aphid species have shown evidence of rapid evolution in response to changes in their environment (Harmon *et al*. 2009), and a similar finding of a greater level of differentiation among aphid populations compared to their parasitoids has been recorded (Nyabuga *et al*. 2012). In the latter study, the authors suspected that metapopulation dynamics constrained the rate of evolution of the parasitoid relative to that of its aphid host due to the lag time in colonization of new patches. This lag time may disrupt the coevolutionary arms race (Lapchin & Guillemaud 2005), and a difference in evolutionary rates of interacting organisms can have negative impacts on the stability of their relationships. For example, predator-prey dynamics can be negatively affected by rapid evolution of the prey species (Yoshida *et al*. 2003), while conversely, biological control of weeds can be negatively affected by rapid evolution of the natural enemy (Smith *et al*. 2010). Therefore, by incorporating metapopulation dynamics into our understanding of the coevolutionary interactions between natural enemies and their hosts, the geographic mosaic theory of coevolution (Thompson 1994, 2005) may help elucidate the factors that influence the sustainability of biological control services. As such, the

walnut aphid biological control program provides a model system for the continued study of factors that may either promote or inhibit coevolutionary interactions between introduced natural enemies and their target pests.

Conclusions

Using the population genomics approach proposed by Vermeer *et al*. (2011), we identified the signatures of a geographic mosaic of coevolution between the walnut aphid (*C. juglandicola*) and the braconid parasitoid *T. pallidus*. Locations representing both coevolutionary hotspots and coevolutionary coldspots were identified, although the number of each changed between F_{ST} and Rho_{ST} as separate measures of population differentiation. Given the localized breakdowns observed in the control of walnut aphids by *T. pallidus* in California (Hougardy & Mills 2008), we believe that this pattern is consistent with expected oscillations between coevolutionary hotspots and coldspots. Future research should address whether hot- or coldspots are associated with breakdowns in biological control, and what management practices could be used to either promote or disrupt these coevolutionary interactions.

Acknowledgments

Funding for this study was made possible by an EPA-STAR Fellowship and a Robert van den Bosch Memorial Fellowship. I would like to thank K. Anderson, and M. Labbé for their field assistance in collecting *T. pallidus* and *C. juglandicola*. In addition, I would like to thank the University of California Cooperative Extension, and the East Bay Regional Parks Department, as well as the individual walnut growers who granted us access to their properties, J. Lozier and B. Ort for their technical assistance in conducting the molecular analyses, and R. Nielsen and G. Roderick for their comments on an earlier draft of this manuscript. I would also like to thank N. Mills for his incredible enthusiasm and the tireless effort he put into this chapter.

Chapter 5. References

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Table 5.1 – Collection locality information for *Chromaphis juglandicola* and *Trioxys pallidus*

Population IDs and locations sampled in this study including the number of females genotyped from each location (*N*), and the mean observed (*Ho*) and expected (*He*) heterozygosity.

ID	Location	Host	Collector(s)	Date	N	Ho	He
T. pallidus							
J0178	Yuba City, CA	*P. juglandis*	J Andersen	27xi2011	7	0.219	0.331
J0179	Modesto, CA	*C. juglandicola*	J Andersen	5vi2012	12	0.344	0.354
J0057	Arbuckle, CA	*C. juglandicola*	J Andersen	19vii2010	14	0.295	0.371*
J0070	Upper Lake, CA	*C. juglandicola*	J Andersen & M Labbé	13ix2010	13	0.277	0.319
J0120	Parnac, France	*C. juglandicola*	J Andersen & M Labbé	2vi2011	14	0.357	0.523*
J0188	Newark, CA	*C. juglandicola*	J Andersen & M Labbé	30viii2012	10	0.347	0.386
C. juglandicola							
A0052	Linden, CA	Walnut	J Andersen	10vii2010	7	0.115	0.141
A0046	Modesto, CA	Walnut	J Andersen & K Anderson	7vii2010	9	0.102	0.097
A0057	Arbuckle, CA	Walnut	J Andersen	19vii2010	12	0.201	0.355*
A0070	Upper Lake, CA	Walnut	J Andersen & M Labbé	13ix2010	9	0.176	0.193
A0120	Parnac, France	Walnut	J Andersen & M Labbé	2vi2011	20	0.079	0.095
A0189	Newark, CA	Walnut	J Andersen & M Labbé	18ix2014	12	0.160	0.167

* Significant departure from HWE ($P < 0.001$)

Table 5.2 – Measures of population differentiation among locations for *Chromaphis juglandicola* and *Trioxys pallidus* based on F_{ST} (below the diagonal) and Rho_{ST} (above the diagonal).

T. pallidus						
	Yuba City	Modesto	Arbuckle	Upper Lake	Parnac	Newark
Yuba City	--	0.015†	0.034	0.003	0.316*	0.007
Modesto	0.030†	--	0.020Δ	0.013†	0.351*	0.002†
Arbuckle	0.024	0.042Δ	--	0.021	0.320*	0.009
Upper Lake	0.014	0.026†	0.014	--	0.370*	0.007
Parnac	0.244*	0.268*	0.273*	0.278*	--	0.312*
Newark	0.019	0.038†	0.025	0.010	0.240*	--

C. juglandicola						
	Linden	Modesto	Arbuckle	Upper Lake	Parnac	Newark
Linden	--	0.100	0.169*	0.046*	0.173	0.125
Modesto	0.027	--	0.099*	0.018*	0.028	0.033
Arbuckle	0.075*	0.122*	--	0.030*	0.126*	0.106*
Upper Lake	0.308*	0.347*	0.215*	--	0.108*	0.051*
Parnac	0.087	0.053	0.255*	0.411*	--	0.070*
Newark	0.008	0.080	0.115*	0.329*	0.181*	--

* Significant after Bonferonni correction for multiple comparisons (adjusted $\alpha = 0.003$)

† $P < 0.05$

Δ $P < 0.01$

Table 5.3 – Measures of recent migration rates for *Chromaphis juglandicola* and *Trioxys pallidus* using BA3

Average migration rates between each population pairs should be read as Row Name –> Column Name. Significant migration rates (i.e., average migration rate ± 2 SE not including zero) are highlighted in bold.

T. pallidus						
	Yuba City	Modesto	Arbuckle	Upper Lake	Parnac	Newark
Yuba City	--	0.026	0.026	**0.202**	0.026	0.017
Modesto	0.018	--	0.018	**0.240**	0.019	0.019
Arbuckle	0.017	0.017	--	**0.250**	0.017	0.017
Upper Lake	0.017	0.017	0.017	--	0.017	0.017
Parnac	0.017	0.017	0.016	0.017	--	**0.188**
Newark	0.021	0.022	0.021	**0.227**	0.022	--

C. juglandicola						
	Linden	Modesto	Arbuckle	Upper Lake	Parnac	Newark
Linden	--	0.026	0.026	0.026	0.026	**0.202**
Modesto	0.023	--	0.023	0.023	0.023	**0.219**
Arbuckle	0.020	0.020	--	0.048	0.020	**0.095**
Upper Lake	0.022	0.022	0.022	--	0.022	0.038
Parnac	0.013	0.013	0.013	0.013	--	**0.269**
Newark	0.018	0.018	0.024	0.023	0.018	--

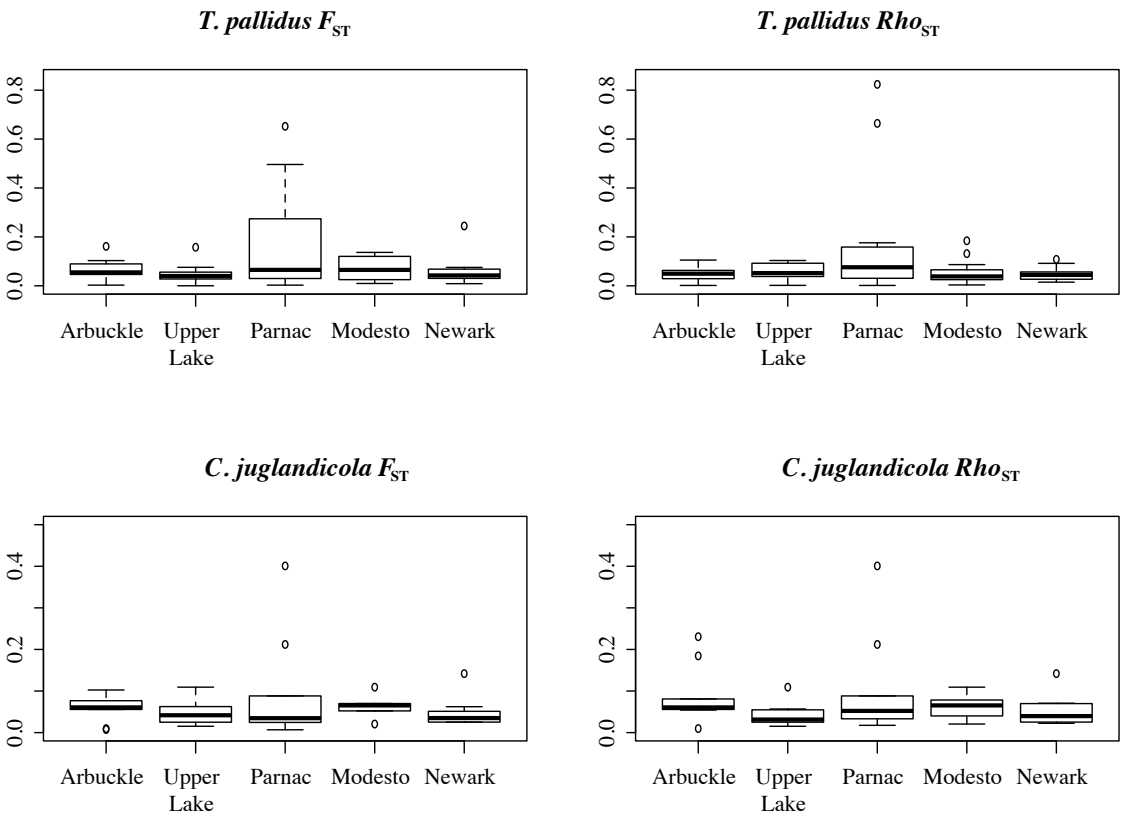

Figure 5.1 – Distributions of locus-specific measures of F_{ST} and Rho_{ST} for different populations of *Chromaphis juglandicola* and *Trioxys pallidus*.
The dark line represents the median measure for each population (compared to the reference), the open box is the interquartile range (IQR) with whiskers extending to 1.5 * IQR, and outlier loci are represented as open circles.

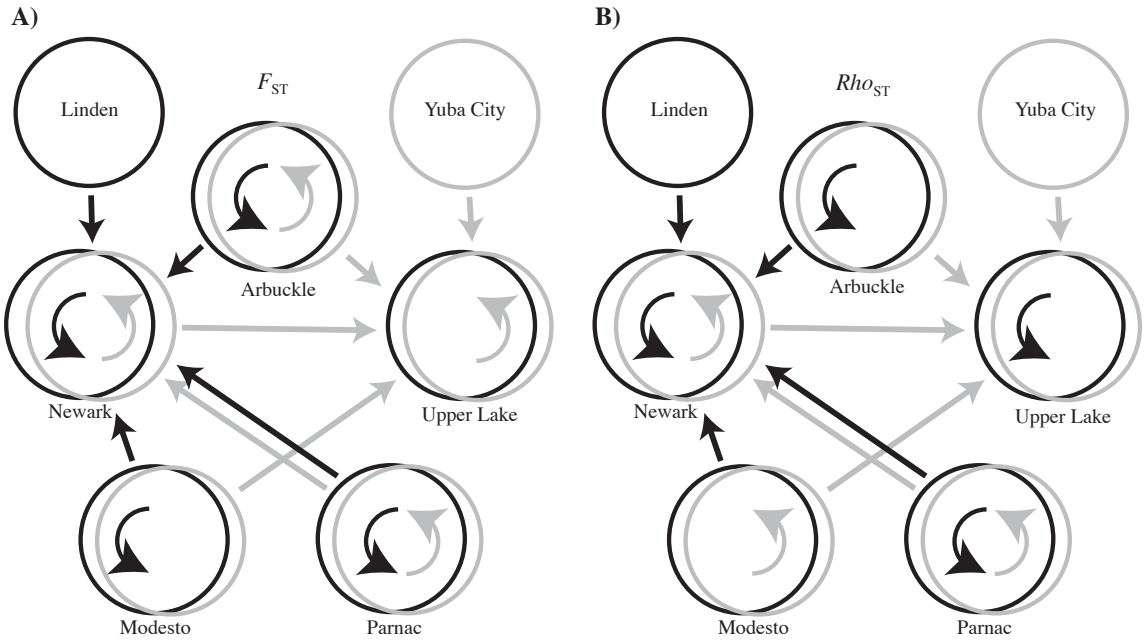

Figure 5.2 – Graphical representation of coevolutionary hotspots and coldspots in the geographic mosaic of coevolution between *Chromaphis juglandicola* and *Trioxys pallidus* based on A) F_{ST} and B) Rho_{ST}.
Grey circles represent locations from which *T. pallidus* was collected, and black circles represent locations from which *C. juglandicola* was collected. Arrows between circles represent the direction of significant geneflow between locations and curved arrows within the circles represent the presence of outlier loci for either *T. pallidus* (grey) or *C. juglandicola* (black). Locations with curved arrows for both species represent potential coevolutionary hotspots, while those locations with curved arrows for only one or neither species represent potential coevolutionary coldspots.

CHAPTER 6: HYBRIDIZATION BETWEEN TWO HOST-ASSOCIATED STRAINS OF KNOTWEED PSYLLID, *APHALARA ITADORI*, LEADS TO PATTERNS OF NON-MATERNAL INHERITANCE OF BOTH PRIMARY AND SECONDARY ENDOSYMBIONTS.

Abstract

Three species of invasive knotweeds (*Fallopia japonica*, *F. sachalinensis*, and *F.* x *bohemica*) cause extensive damage to riparian and roadside habitats in North America. Currently, two strains of the psyllid *Aphalara itadori* are being evaluated for introduction into the United States and Canada for the biological control of these knotweeds after *A. itadori*'s successful introduction into the United Kingdom. If approved for release, hybridization between individuals from these two strains is likely and understanding whether barriers to hybridization exist could have an important impact on the sustainability of this biological control program. Here we developed two single nucleotide polymorphism (SNP) arrays and examined their utility for identifying individuals of known pure (Hokkaido and Kyushu) and hybrid origins. We found that one array (labeled the "full array") correctly identified all individuals to pure and hybrid classes whereas a second array (labeled the "reduced array") was able to correctly identify most pure individuals, but not hybrids. While we found no barriers to hybridization, we found a curious pattern of potential horizontal transmission of the primary endosymbiont *Candidatus Carsonella rubii* and the secondary facultative endosymbiont *Sodalis* sp. in one of our hybrid crosses. We discuss potential mechanisms for the horizontal transmission of *Carsonella* and *Sodalis* and compare our results to recent studies of host preference and fitness of these biological control agents.

Introduction

Hybridization in nature was long considered a relatively rare event with both pre- and/or post-zygotic barriers preventing introgression of genomic material between species (Kirkpatrick 2000; Orr and Presgraves 2000; Presgraves 2002; Lukhtanov *et al*. 2005). Yet, increasingly it is being recognized that hybridization between closely-related organisms occurs within a broad range of taxonomic groups (Allendorf *et al*. 2001) and that hybridization between genetically distinct lineages has been identified as one of the leading causes of evolution and speciation (Mallet 2005). These hybridization events can lead to shifts in host-preference (Feder *et al*. 2003) and host-associated speciation events (Schwarz *et al*. 2005); furthermore, the rate at which hybridization events occur has undoubtedly been increased through anthropogenic activities that accidentally facilitate encounters between allopatric lineages (Allendorf *et al*. 2001).

Among closely-related insect taxa, hybridization appears to be a common occurrence (Schwenk *et al*. 2008), and speculation of hybridization events between insect and non-insect taxa also exists in the literature (e.g. Williamson 2009). Given the extensive use of insect species as natural enemies in classical biological control programs that seek to suppress the abundance of exotic pests and noxious weeds through the deliberate introduction of specialist natural enemies (Hoddle 2004; Van Driesche *et al*. 2010), hybridization has also likely played a frequent, but poorly known, role in the

sustainability of biological control services. Historically, many programs were established by collecting individuals of a selected natural enemy species from different localities and/or hosts within its region of origin, which have been referred to as strains, biotypes, and/or ecotypes (Clarke & Walter 1995; Heraty 2009). This practice is much less common today, however, due to the recognition that each strain needs to be tested separately for its host specificity (Hopper *et al*. 2005). From an evolutionary perspective, strains likely represent an early point in the process of speciation (i.e. prior to genetic isolation [Dres & Mallet 2002]) and can be influenced by factors such as geneflow, genetic diversity, and phenotypic plasticity (Ruiz-Montoya & Nunez-Farfan 2013). How frequently these strains hybridize, or what effect hybridization has on the sustainability of biological control services is unclear as relatively few studies have examined the effects of post-introduction hybridization. Those laboratory studies that have examined the potential effects of hybridization between strains have found conflicting evidence of both reduced (e.g. Messing & AliNiazzee 1988) and increased (e.g. Szűcs *et al*. 2012) fitness of the hybrids relative to their parents.

While hybridization may be common among closely-related insect taxa, incompatabilities among nuclear or cytoplasmic loci (Dobzhansky 1936; Hurst & Pomiankowski 1991), or incompatibilities between cytoplasmic loci and either nuclear loci or sex chromosomes (Hurst & Pomiankowski 1991; Ellison *et al*. 2008) can determine whether hybridization is possible within particular taxonomic groups. For insects, these incompatabilities are frequently the result of sex-ratio distorting secondary endosymbionts (Turelli 1994; Bordenstein *et al*. 2001). Endosymbiotic bacteria, including species of *Wolbachia, Cardinium, Rikettsia, Spiroplasma* and *Arsenophagus*, are commonly found in insects (Engelstaedter & Hurst 2009), with *Wolbachia* alone being estimated to infect over 65% of insect species (Hilgenboecker *et al*. 2008). The effects of these reproductive manipulators are diverse, but can be grouped into two broad categories; 1) sex ratio distortion (e.g. male-killing, feminization of males, or parthenogenesis induction); and 2) conditional sterility via cytoplasmic-incompatibility (Ferrari & Vavre 2011); however, to date only *Wolbachia* and *Cardinium* are known to cause cytoplasmic incompatability in arthropods (Engelstaedter & Hurst 2009). In classical biological control programs, reproductive manipulators may have important consequences for the establishment and impact of introduced natural enemies as their presence may prevent geneflow between populations (Cheyppe-Buchmann *et al*. 2011) and/or influence patterns of host specialization (Branca *et al*. 2011). Therefore, it is important not only to examine the population structure of target pests (or weeds) and candidate natural enemies, but also to characterize the microbial diversity present in natural enemy "strains" for their potential influence on hybridization and host specialization.

One system that provides a valuable model for testing the potential effects of hybridization on the host specialization and performance of a natural enemy in a classical biological control setting is the proposed program for the biological control of invasive knotweeds (*Fallopia* spp.) in North America. These invasive weeds include Japanese (*F. japonica*), giant (*F. sachalinensis*), and Bohemian knotweed (*F.* x *bohemica*), and have been introduced to North America and Europe on multiple occasions beginning in the

1840s (Beerling *et al*. 1994). Invasive species of knotweed occur along riverbanks and roadways and in wetlands and disturbed areas where they invade riparian communities (Maerz *et al.* 2005; Siemens & Blossey 2007), and reduce native plant and insect species diversity (Gerber *et al.* 2008; Murrell *et al.* 2011).

The psyllid *Aphalara itadori* Shinji was recently released in Europe for the biological control of knotweeds (Shaw *et al.* 2009; Djeddour & Shaw 2010), and in part because of the success of this program, it has also been proposed for introduction into the United States and Canada. Because of regional differences in the abundance of each species of knotweed (Gaskin *et al*. 2014) two strains of *A. itadori* that differ in their fitness among *Fallopia* species have been proposed for introduction (Grevstad *et al*. 2013). One strain performs best on Japanese and Bohemian knotweeds and originates from the Japanese island of Kyushu – this strain was released in the UK for the biological control program there (Shaw *et al.* 2009) – while the other strain performs best on giant knotweed and originates from the Japanese island of Hokkaido (Grevstad *et al.* 2013). Due to overlapping distributions of the invasive species of knotweed in the United States and Canada, the potential for hybridization between the released strains of psyllid is expected to be high. As there are no known morphological differences between the two psyllid strains, neutral molecular markers are needed to be able to distinguish them post introduction and to detect the incidence of hybridization. In addition, psyllids are known to harbor both primary (*Candidatus Carsonella ruddii*, hereafter *"Carsonella"*) and secondary (*Sodalis* sp., hereafter "*Sodalis*") endosymbionts (Baumann 2005). Thus it may also be important to examine whether endosymbionts influence patterns of host plant association, efficacy as natural enemies, and the ability of individuals from each strain to hybridize. Therefore, the objectives of this study were 1) to develop neutral molecular markers for the two *A. itadori* strains, 2) to test the ability of these markers to successfully differentiate pure and hybrid individuals of *A. itadori*, and 3) to test whether there are barriers to the transmission of primary and secondary endosymbionts between strains of *A. itadori*.

Methods

Laboratory Colonies

Laboratory colonies of the Hokkaido and Kyushu strains of *A. itadori* were established in the Insect Quarantine Facility at Oregon State University and the Lethbridge Research Center Quarantine Facility at Agriculture and Agri-Food Canada. Individuals from the Hokkaido strain were collected by Robert S. Bourchier and Fritzi S. Grevstad from giant knotweed on Hokkaido in July of 2007. Individuals from the Kyushu strain were obtained from a colony established and released in the U.K. by Dr. Richard H. Shaw in November of 2010. Reciprocal hybrid crosses were then performed using males and females from each strain of *A. itadori* and established on giant, Bohemian, and Japanese knotweeds at the Lethbridge Research Center Quarantine Facility at Agriculture and Agri-Food Canada.

DNA Extractions for Identification of A. itadori Strains

DNA was extracted from individuals of each strain of *A. itadori* from colonies maintained at the Insect Quarantine Facility at Oregon State University, as well as from each hybrid class (Hokkaido female x Kyushu male [Hf*Km] and Kyushu female x Hokkaido male [Kf*Hm]) from colonies reared on Japanese, giant, and Bohemian knotweed at the Lethbridge Research Center Quarantine Facility at Agriculture and Agri-Food Canada. Extractions were performed using the Qiagen Gentra Puregene Kit (Qiagen Co.) following manufacturer's recommendations, except for the following modifications. First, the cell lysing step was performed using an incubation temperature of 55°C for a minimum of 48 h; and second, because of the low expected yields due to the small size of the psyllids, we pelleted the DNA using a prolonged centrifuge step of 13,000 rpm for 10 min, and cleaned the DNA pellet with two separate 70% ethanol washes. DNA yields were estimated using a ND-1000 NanoDrop® (NanoDrop Technologies, Inc.) and concentrations of double stranded DNA were measured using the Qubit® dsDNA HS Assay kit (Life Technologies Corp.).

Mitochondrial DNA Analyses

As a preliminary indicator of the amount of genetic variation present between the two strains of *A. itadori*, we examined the amount of sequence variation in the "Barcoding" region of the mitochondrial gene Cytochrome Oxidase I (COI) for two individuals of each strain. PCR reactions were carried out on a BioRad Dyad programmable thermocycler (BioRad Laboratories, Inc.) using the primer pair LCO and HCO (Folmer *et al*. 1994) and followed the conditions presented by Hebert *et al*. (2003). Promega GoTaq DNA polymerase, buffers, and dNTPs (Promega, Corp.) were used at the following concentrations; 5 µl of Promega GoTaq buffer, 0.5 µl of dNTP, 0.5 µl of both forward and reverse primers, 0.2 µl of Promega GoTaq taq polymerase, 1 µl of DNA, and sufficient HPLC purified H_20 added to bring the final volume to 25 µl. PCR products were held at 17°C before being visualized on a 1.5% agarose gel. DNA sequencing of both forward and reverse fragments was performed at the DNA Sequencing Facility at the University of California Berkeley, and sequence results were visualized and edited using Geneious Pro v. 5.6.2 (Drummond *et al*. 2012).

Next-generation Sequencing

To achieve sufficient genomic material for Next-generation Sequencing (NGS), ten individuals were pooled for both the Hokkaido and Kyushu strains of *A. itadori* and placed into separate labeled 1.5ml micro-centrifuge tubes. Genomic DNA was extracted from both of the pooled samples using the Qiagen Gentra Pure Gene DNA Extraction Kit (Qiagen, Co.) following the manufacturer's instructions. Sequencing libraries for each pooled extract were then created using the PrepX™ ILM DNA Library Kit (Wafergen Biosystems, Inc.) at the Functional Genomics Laboratory at the University of California Berkeley, and each library was constructed using a different Illumina (Illumina, Inc.) barcoding primer. Sequencing libraries were examined for fragment length distribution and concentrations using a 2100 Expert Bioanalyzer (Agiliant Technologies), and a KAPA Biosystems Library Quantification Kit (KAPA Biosystems). The two libraries were then combined and sequenced using a single run of an Illumina HiSeq2000

(Illumina, Inc.) sequencer at the Vincent J. Coates Genomics Sequencing Laboratory, University of California, Berkeley.

Low quality Illumina sequencing reads were removed and summary statistics were calculated using the FASTX-Toolkit (Gordon 2014). The Illumina sequencing reads that passed the fastq_quality_filter were then assembled into contigs using the *de novo* assembly program Velvet 1.1.06 (Zerbino & Birney 2008) with a k-mer length of 67. To determine the genomic origin for each of the assembled contigs we compared them to sequences in the NCBI GenBank Database (Benson et al. 2013) using the blast-n toolkit (Altschul et al. 1990). Any contig with an E-value > 0.05 was considered unidentified. For contigs with an E-value ≤ 0.05 the single top-match sequence was used to classify them as either of insect, microbial (Bacteria and/or Archaea), viral, or "other" origin.

SNP Array Design and Genotyping

To identify polymorphic sites for subsequent genotyping analyses, we used the software package SAMtools (Li *et al.* 2009) to map the individual Illumina sequence reads to our *de novo* contig assemblies, and to summarize the number, locations and quality scores for polymorphic sites. The resulting ".vcf" report was then filtered to remove contigs that included multiple polymorphic sites, polymorphic sites receiving a quality score < 100, and polymorphic sites identified as indels. Using this list of candidate SNPs, two SNP arrays were then designed using Sequenom's Online Assay Design Suite v 1.0 (Sequenom Inc.) following the manufacturer's instructions with default settings, and assembled by the Genotyping Facility at the University of Arizona. A first array (hereafter the "full array") included 141 SNPs chosen based on their quality scores from the .vcf report, and a second array (hereafter the "reduced array") included a subset of those SNPs from the first array (29 SNPs). The reduced array was designed to include only those SNPs that were associated with contigs that matched published insect, *Carsonella*, and *Sodalis* sequences. The full array was then used to genotype 95 individuals (27 each of the pure Hokkaido and Kyushu strains reared on giant and Japanese knotweeds, respectively, and 20 Hf*Km and 21 Kf*Hm hybrids reared on giant knotweed). The reduced array was then used to genotype an additional 95 individuals (12 pure Hokkaido reared on giant knotweed, 23 pure Kyushu reared on Japanese knotweed, 13 Hf*Km and 24 Hf*Km hybrids reared on Japanese knotweed, and 23 Hf*Km hybrids reared on Bohemian knotweed). Genotyping was conducted on a Sequenom MassArray® MALDI-TOF (Sequenom Inc.) at the Genotyping Facility at the University of Arizona where DNA concentrations were normalized prior to analysis.

Population Structure and Hybridization Analyses

Because the genotyping included SNPs from the endosymbionts *Carsonella* and *Sodalis*, prior to analyses we concatenated the SNPs for both species and rescored them as haplotypes so that only two loci (one for each endosymbiont species) would be included in subsequent analyses. For those individuals of *A. itadori* genotyped using the full array, we then estimated their probability of assignment (Q) to distinct genetic clusters, and the optimal number of genetic clusters represented by individuals in the

dataset for a potential number of clusters (K) of 1 through 10 using the program fastStructure (Raj *et al*. 2014). For K=2 we assigned individuals as "pure" if they received a score of Q≥0.8 to one of the two clusters, or as a "hybrid" if they received a score of Q<0.8 (Havill *et al*. 2012). We also estimated the probability of assignment (Z) that an individual was of parental, F1, F2, or backcrossed origin (however, we combined the assignment probabilities from all the hybrid classes to present a single Z value for hybrid status) with the software program NewHybrids (Anderson & Thompson 2002; Anderson 2008). We used four independent runs of 10 million generations each, random starting values, uniform priors for the estimates of theta and pi, and a burn in period of 1 million generations. These same methods were then used to estimate the probability of assignment for individuals genotyped with the reduced array. In this case, because the SNPs used in the reduced array were also represented as a subset of those from the full array, we combined the genotyping results from both arrays and removed all SNPs that were unique to the full array before analysis.

Results

Mitochondrial DNA Analyses

We sequenced a 678-basepair fragment of mitochondrial locus COI from both the Hokkaido and Kyushu strains of *A. itadori*. Within strains of *A. itadori* there were no basepair differences, however there were four basepair differences (all represented third codon position synonymous mutations) between the strains. One basepair difference, position 249 of our alignment, is a binding site for two commonly used restriction enzymes (HpaII and MspI). Consequently, this binding site could be used to differentiate individuals by maternal origin as the use of these restriction enzymes will cut the Hokkaido sequence into two fragments (one of ~ 250 bp and the other of ~ 430 bp), but will not cut the Kyushu sequence. Sequences of COI from the two strains of *A. itadori* can be found on GenBank with Accession Numbers KP113670-KP113673.

Next-generation Sequencing

Our Illumina HiSeq run resulted in 71,390,662 and 83,941,610 100 bp reads for the Hokkaido and Kyushu libraries, respectively, of which 66,392,260 and 77,812,449 passed the fastq_quality_filter. Raw sequence reads for both strains can be found at NCBI BioSample (Accession Number SUB724178). The full list of assembled contigs with E-values < 0.05 can be found in Appendix Table 1, along with the single top-match sequence in the NCBI GenBank Database and its likely genomic origin (e.g. insect, microbial, viral, or other). In total we constructed 21,177 contigs with an average length of 731 bp and an average coverage of 9.5x. Of these, 13,360 contigs were unidentified, 4,961 were classified as of insect origin, 1,375 as of microbial origin (i.e. either Bacteria or Archaea), 39 as of viral origin, and 2,042 as of other origins. Only three of 21,177 contigs matched a known reproductive manipulator (*Wolbachia*), therefore the presence of reproductive manipulators in this system seems unlikely.

Among these contigs we identified 133,244 polymorphic sites using SAMtools that included 125,915 SNPs and 7,329 indels, of which 560 potential SNP markers passed our quality filters. From these 560 potential SNP markers, 141 SNPs were identified for

genotyping analyses based on their quality scores and their compatibility for multiplexing. A list of these 141 SNPs, their expected polymorphic states, and 300 bp of FASTA formatted sequence data in both 5' and 3' directions are presented in Appendix Table 2.

SNP Array Design and Genotyping

All individuals were successfully genotyped in the full array, and of the 141 SNPs included, 119 were amplified in more than 50% of the *A. itadori* samples. Thirty-eight of these represented SNPs from contigs that matched published insect genomic data, 80 from contigs that matched microbial genomic data, and one from a contig that matched viral genomic data. Of those SNPs from contigs that matched microbes, the majority (60) matched sequences from the secondary facultative endosymbiont *Sodalis*, and four matched sequences from the primary endosymbiont *Carsonella*. Ninety-two of 95 individuals were successfully genotyped in the reduced array, and of the 29 SNPs included, 24 were amplified in more than 90% of individuals, one was amplified in more than 60% of individuals, and the remaining four SNPs failed to amplify. These SNPs represented a subset of those from the full array, and included 13 from contigs that matched insect genomic data, three from contigs that matched sequences for *Carsonella,* and nine from contigs that matched sequences for *Sodalis*. The results of our SNP genotyping runs for the full and reduced arrays are presented in Appendix Tables 3 and 4, respectively.

Population Structure and Hybridization Analyses

Prior to analyses, the genotyping results from each array were then trimmed to include only those SNPs that were successfully genotyped in > 75% of individuals (Appendix Tables 3 and 4). The results from our fastStructure analyses of the full array indicated that the optimal number of genetic clusters represented in this dataset was K = 4. The probabilities of assignment for each individual at K = 2 through K = 4 can be seen in Figure 6.1. At K = 2, fastStructure correctly assigned all individuals as either pure or hybrids. At K = 3 and K = 4, fastStructure correctly assigned all but two individuals from the Hokkaido and Kyushu strains as pure, and all hybrid individuals were either assigned to a third genetic cluster (at K = 3) or two additional genetic clusters (at K = 4) with Q > 0.8. Similarly, NewHybrids correctly assigned individuals to pure and hybrid classes with Z ≥ 0.8 (Appendix Figure 2).

The results from our fastStructure analyses of the reduced array indicated that the optimal number of genetic clusters represented in this dataset was K = 2. The probability of assignment (Q) for each individual at K = 2 through K = 4 can be seen in Figure 6.2. At K = 2, 36 of the 38 individuals from the Hokkaido strain and 42 of 50 individuals from the Kyushu strain were correctly assigned as pure individuals of their respective strains, 57 of the 99 hybrids individuals were correctly classified as hybrids, and one individual from the Hokkaido strain was miss-assigned to the Kyushu strain (Q = 0.82). As this individual was the only pure individual miss-assigned to the wrong strain in both datasets, it likely represents a mislabeled DNA extract. In contrast to the analysis of the full array, while NewHybrids correctly assigned all pure individuals (except for our likely

mislabeled extract) with the probability of assignment ($Z > 0.95$; Appendix Figure 3) it failed to correctly identify hybrid individuals and assigned all hybrids to either the pure Hokkaido or Kyushu strains with high probabilities of assignment ($Z > 0.95$).

Endosymbiont Haplotypes

In our analysis of the full array, all individuals from the Kyushu strain had a single *Carsonella* haplotype (labeled *Ca* haplotype 1) and all individuals from the Hokkaido strain had a different *Carsonella* haplotype (labeled *Ca* haplotype 2; Appendix Table 3). Curiously, all hybrid individuals also had *Ca* haplotype 2. For *Sodalis*, each strain had individuals with a strain-specific haplotype (labeled *So* haplotype 1 for Kyushu [n=23] and *So* haplotype 2 for Hokkaido [n=12]). In addition four individuals from Kyushu and 14 individuals from Hokkaido had an additional *Sodalis* haplotype labeled *So* haplotype 3. For one individual from Hokkaido it was unclear whether this individual had *So* haplotype 2 or 3 because SNP062, which differentiates these two haplotypes, failed to amplify. SNP062 also failed to amplify in a large proportion of individuals (~ 40%) from the reduced array (Appendix Table 4), therefore when analyzing the reduced array we combined *So* haplotypes 2 and 3 into a single haplotype group (labeled *So* shared haplotype; Figure 6.2). Based on these results, all individuals from the Kyushu strain, all Kf*Hm hybrids from Japanese knotweed, and the likely mislabeled Hokkaido individual had *Ca* haplotype 1. All but four of these individuals (all from the Kyushu strain) also had *So* haplotype 1. The four remaining individuals from the Kyushu strain, all individuals from the Hokkaido strain and all other hybrids had *Ca* haplotype 2 and *So* shared haplotype.

Discussion

Long-term monitoring is typically missing from most classical biological control programs for pests and weeds (Mills 2000; McCoy & Frank 2010). For programs where multiple strains of a natural enemy have been introduced, the long-term effects of hybridization between the previously distinct strains is unclear, but laboratory evidence suggests the potential for either reduced fitness through hybrid breakdown (Messing and AliNiazee 1988; Bean *et al*. 2013) or increased fitness through hybrid vigor (Szűcs *et al*. 2012). While hybridization has been observed to be one of the leading causes of evolution and speciation (Mallet 2005), long-term monitoring of hybridizing populations has been difficult to implement due to the large numbers of independent loci required for accurate assignment of individuals to pure or hybrid classes (Vaha & Primmer 2006). Using NGS technologies, we developed and validated two SNP arrays to distinguish between pure and hybrid individuals of two strains of *A. itadori*. Based on a comparison of assignment accuracy between these two arrays using frequently used assignment methods (fastStructure [Raj *et al*. 2014], and NewHybrids [Anderson & Thompson 2002; Anderson 2008]), we found that our full array correctly identified all pure and hybrid individuals. In contrast, while the reduced array correctly identified the majority of pure individuals this array failed to identify individuals of hybrid origin using either fastStructure or NewHybrids. Therefore, post-release surveys of *A. itadori* should use, as a minimum, the SNPs found in the full array.

Patterns of endosymbiont horizontal transmission

One curious result from these analyses is the potential horizontal transmission of the primary endosymbiont *Candidatus Carsonella ruddii* during the Kf*Hm hybrid cross reared on giant knotweed. We did not, however, directly test for horizontal transmission during our study, and thus we cannot rule out the hypothesis that there are individuals of the Kyushu strain that harbor the Hokkaido *Carsonella* haplotype (*Ca* haplotype 2), but at low enough frequencies that we did not detect them in our surveys. However, given our sample sizes (n = 38 for the Hokkaido strain and n = 50 for the Kyushu strain), this seems unlikely. What mechanisms allowed for this apparent horizontal transmission of the primary endosymbiont *Carsonella* are unknown. Among plant feeding insects, and Hemiptera in particular, primary endosymbionts are thought to be maternally inherited (Baumann 2005) and yet numerous ancient horizontal transmission events have been documented (Moran *et al*. 2005; Conord *et al*. 2008; Toju *et al*. 2013). In some host species symbionts have even shown repeated patterns of horizontal transmission (Duron *et al*. 2014). These events for primary endosymbionts have taken place over evolutionary time scales whereas facultative secondary endosymbionts, such as *Sodalis*, have long been known to show patterns of horizontal transmission on ecological time scales (Baumann 2005). For example, the secondary symbiont *Rickettsia* has been observed to be transferred horizontally from infected to uninfected whiteflies using plants as a vector (Caspi-Fluger *et al*. 2012), and transferred vertically through sperm for the leafhopper *Nephotettix cincticeps* (Watanabe *et al*. 2014). Other secondary symbionts, *Hamiltonella defensa* and *Regiella insecticola*, have also been shown to be transmitted horizontally by parasitoids using "dirty needle" ovipositors (Gehrer & Vorburger 2012). Previous studies have shown that *Sodalis* seems to be particularly capable of horizontal transmission (Russell *et al*. 2003; *Toju et al*. 2013; Duron *et al*. 2014). However, in many insect hosts secondary endosymbionts co-occur with the primary endosymbiont in bacteriocytes (Kliot *et al*. 2014). Therefore, while *Carsonella* has been associated with psyllids for 100-250 my (Baumann 2005), if the *Sodalis* lineages associated with the Hokkaido strain are prone to horizontal transmission, perhaps some type of "piggybacking" during their transmission could lead to the transfer of *Carsonella* as well, though the mechanisms for this need to be explored further.

Why horizontal transmission appears to have occurred during hybrid crosses reared on giant knotweed, and not for those reared on Japanese or Bohemian knotweeds is unclear. However, our hybrid colonies were established by placing females and males of both parental strains in cages, and the colonies were thus established over several generations. It is possible, therefore, that if either the *Sodalis* or the *Carsonella* haplotypes associated with the Hokkaido strain confer fitness benefits to their hosts when feeding on giant knotweed, these haplotypes would increase in frequency while the non-advantageous haplotypes would be purged during subsequent generations. This seems strikingly similar to the pattern we observed for the secondary endosymbiont *Sodalis* in our Kf*Hm hybrids reared on giant knotweed. The shared *Sodalis* haplotype (i.e. *So* haplotype 3 in the full array or *So* shared haplotype in the reduced array) was found at low frequency in the Kyushu strain (~ 8%), yet all hybrid individuals from the Kf*Hm cross on giant knotweed had this haplotype. Given its low starting frequency strong fitness benefits would be required for such a rapid fixation of haplotypes, and Grevestad

et al. (2013) recently showed that such a fitness benefit may exist in this system, when they noted a five-fold difference in survival of the Hokkaido and Kyushu strains on preferred versus non-preferred hosts. They also found that F1 individuals of the Hokkaido strain were half as abundant on giant knotweed, their preferred host, compared to the Kyushu strain on Bohemian knotweed, one of its preferred hosts. Given the low level of genetic divergence between the two strains (< 0.5% divergence at COI), the presence of strain-specific primary and secondary endosymbionts, and the apparent severe fitness effects of being reared on alternative host plants, it is possible that the endosymbionts were responsible for the different host-associations observed for these strains. This scenario would be similar to observations from whiteflies where individuals infected with *Rickettsia* show increased fecundity and survival, and produce more daughters (Himler *et al*. 2011).

Conclusion

In this study we developed two SNP arrays for differentiating two strains of the knotweed psyllid, *A. itadori*, a candidate biological control agent currently being reviewed for release in North America to reduce the abundance of invasive knotweeds. We found that one of these arrays (labeled the full array) was able to accurately identify individuals of both pure and hybrid origin. In addition, while we found no barriers to hybridization between the strains, we did find a curious pattern where hybrid individuals reared on giant knotweed had endosymbiont haplotypes that were representative of the Hokkaido strain regardless of whether their mother was from the Hokkaido or Kyushu strain. Further investigation of the mechanisms by which these endosymbionts are transmitted is needed as this may represent the first known instance of horizontal transmission of a primary endosymbiont on an ecological time scale. The rapid fixation of the shared *Sodalis* haplotype suggests that this secondary endosymbiont may play an important role in host plant preference and subsequent fitness.

Acknowledgements

I would like to thank Raymond Wilson and Karma Tiberg for their laboratory assistance, Justin Choi and Karen Lundy for their assistance in producing the Illumina Sequencing Libraries, and Colleen Ramsower and Crystal Richt for their assistance in designing the SNP arrays and in performing the genotyping runs. I would also like to thank Nichoals Mills, Rasmus Nielsen, and George Roderick for their constructive comments on an earlier version of this chapter. Funding for this study was provide by USDA Forest Service Cooperative agreement grants to Roy Van Driecshe (13-CA-11420004-049), and Fritzi S. Grevstad (10-CA-11420004-255), a Washington State Department of Agriculture grant awarded to Fritzi S. Grevstad (K1227), an and Agriculture and AgriFoods Canada research grant awarded to Robert S. Bourchier, and a Robert van den Bosch Memorial Fellowship awarded to JCA.

Chapter 6. References

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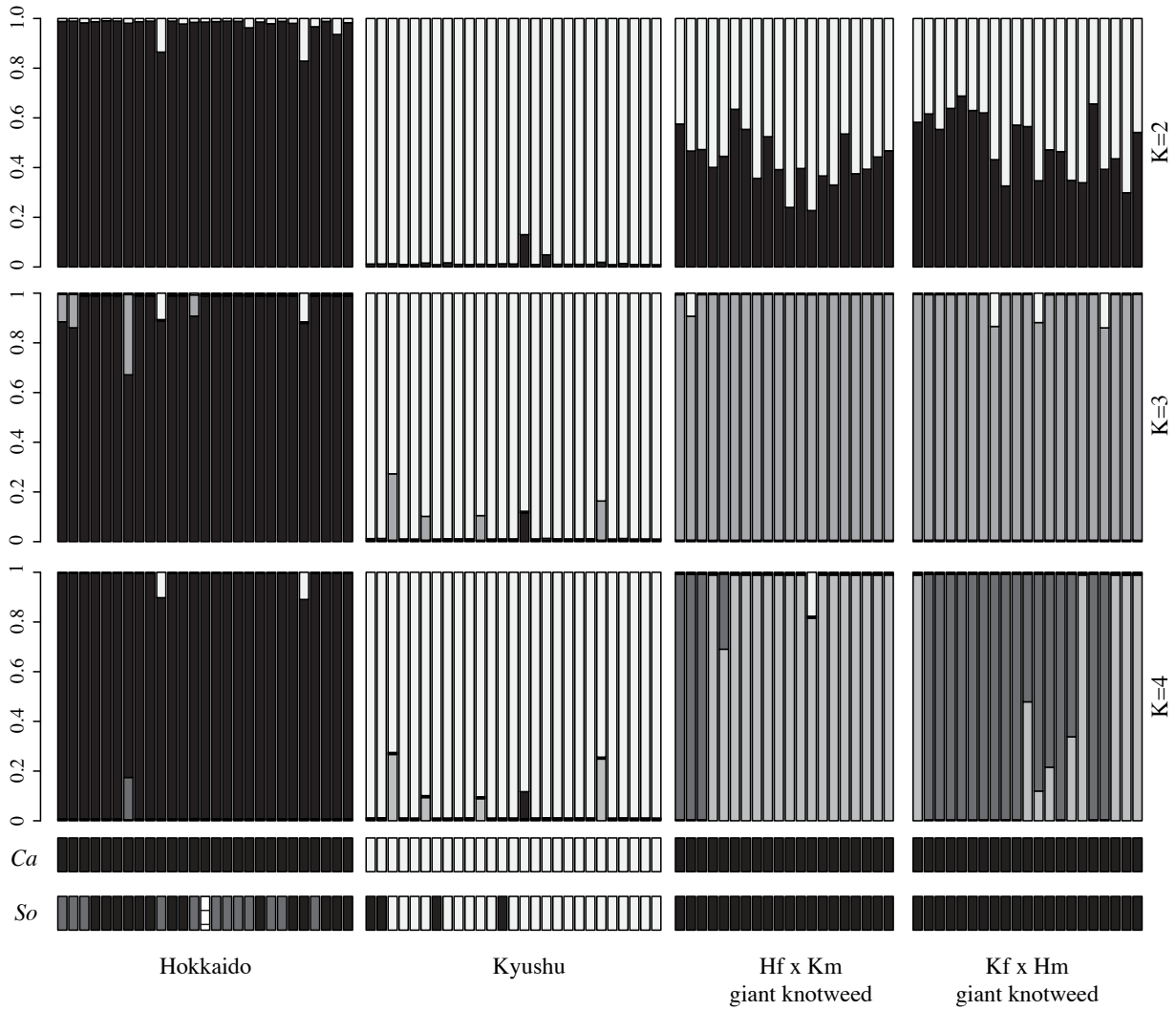

Figure 6.1 – Probability of assignment of each individual of *A. itadori* from the full array to two (K = 2), three (K = 3), and four (K = 4) genetic clusters using fastStructure. Each column represents the proportional assignment of individuals to one of up to four genetic clusters. The two rows below represent the haplotypes of both the primary endosymbiont *Carsonella* (*Ca)* (white = *Ca* haplotype 1, black = *Ca* haplotype 2) and the secondary endosymbiont *Sodalis* (*So*) (white = *So* haplotype 1, gray = *So* haplotype 2, black = *So* haplotype 3, or horizontal lines = undetermined) for each individual. We have divided the graphs to more clearly show the strains of origin.

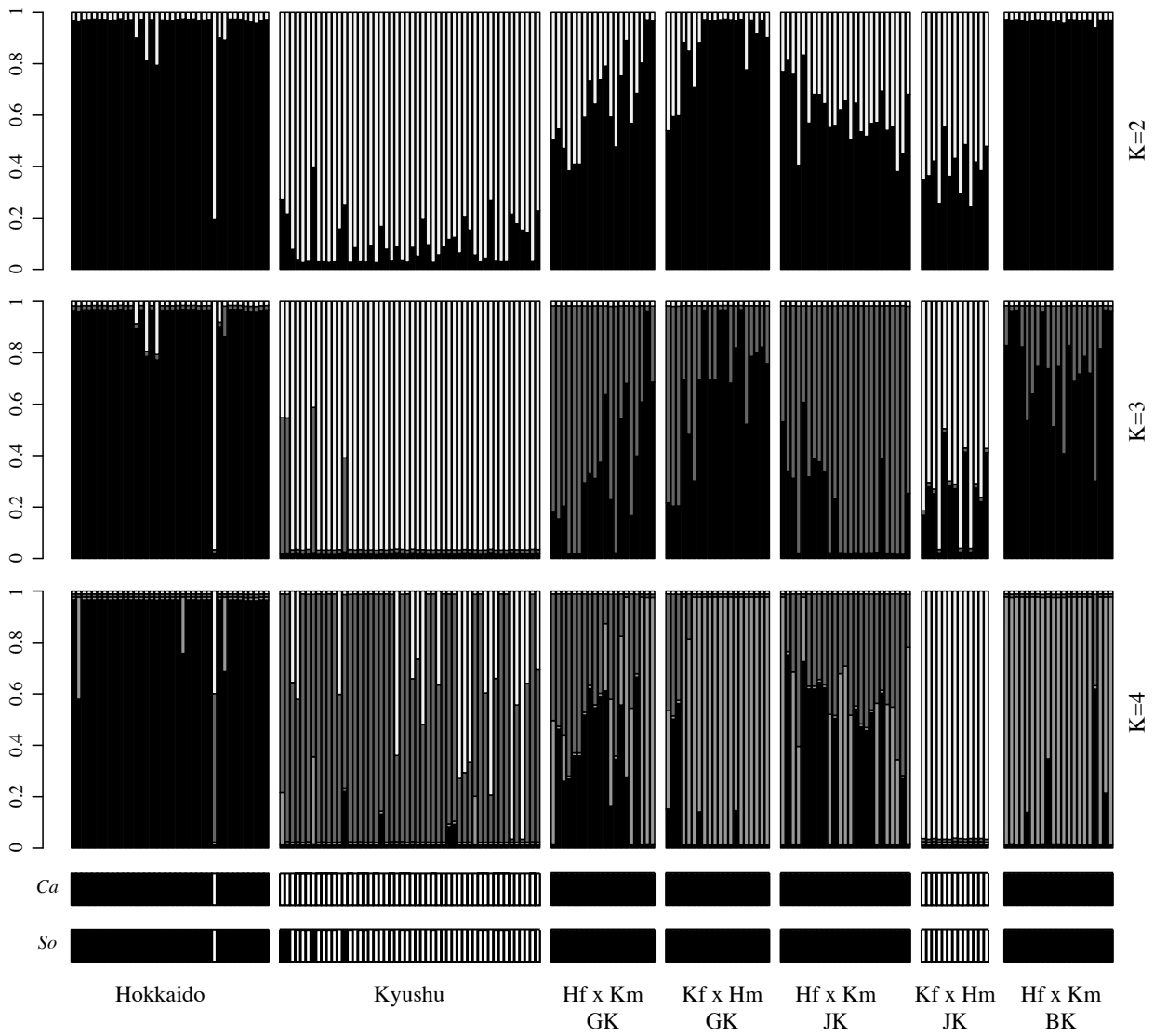

Figure 6.2 – Probability of assignment of each individual of *A. itadori* from the reduced array to potential genetic clusters using fastStructure as per Figure 6.1. Each column represents the proportional assignment of individuals to one of up to four genetic clusters. The two rows below represent the haplotypes of both the primary endosymbiont *Carsonella* (*Ca)* (white = *Ca* haplotype 1, black = *Ca* haplotype 2) and the secondary endosymbiont *Sodalis* (*So*) (white = *So* haplotype 1, black = *So* shared haplotype) for each individual. GK = giant knotweed, JK = Japanese knotweed, and BK = Bohemian knotweed.

CHAPTER 7: CONCLUSIONS

Dissertation Synthesis

In this dissertation I expanded upon our knowledge of the utility of population genetic approaches for the study of the evolution of introduced biological control agents and their target pests. If classical biological control methods (Van Driesche *et al.* 2010) are to provide sustainable pest management services (Bianchi *et al.* 2006; Bale *et al.* 2008) then long-term studies are necessary (Mills 2000; McCoy & Frank 2010; Cory & Franklin 2012). I believe these long-term studies should also include the use of population genetic approaches, and while these approaches have been used successfully in post-release monitoring efforts (Agboton *et al.* 2011; Kyei-Poku & Johny 2013), relatively few studies have examined long-term changes in the efficacy of biological control programs. One such study found that an introduced biological control agent became less efficient at controlling its target pest due to maladaptation (Hufbauer 2002), another found evidence of local adaptation over a 10 year period (Phillips *et al.* 2008), while another found evidence of adaptation to a novel host (Vorsino *et al.* 2014). Given these three very different results, further research is undoubtedly required to better understand the evolutionary trajectories of introduced biological control agents and their target pests.

Because many established biological programs likely were not set up for long-term monitoring, museum voucher specimens may provide an invaluable resource for the examination of post-release evolutionary changes of biological control agents and their target pests. For example, Vorsino *et al.* (2009) used museum specimens to examine changes in the genetic composition of parasitoid wasps used in an augmentative biological control program in the Hawaiian Islands. These authors used a minimally invasive approach (they removed a leg from each specimen), but found that due to DNA degradation they were unable to amplify many of their target loci. I was therefore curious as to whether non-destructive techniques could be used to obtain useable genomic material from museum specimens of parasitoid wasps, and what the barriers to obtaining that material would be. In Chapter 2, I found that the age of the specimen was the most important determinant for the amplification of PCR products, with nuclear loci having a higher probability of amplification from older specimens than mitochondrial loci (Table 2.3). I also found that by sequencing short fragments of mitochondrial DNA, I was able to differentiate voucher specimens of different strains of the biological control agent *Trioxys pallidus* (Figure 2.4 part C) and I was able to confirm the identification of an unknown parasitoid reared from the invasive light brown apple moth (Figure 2.4 part B). While voucher specimens have not been a requirement for biological control programs in all locations (Barratt *et al.* 2010), the results from Chapter 2 indicate that, when present, useful genetic material can be obtained from voucher specimens without conferring external morphological damage.

In addition to voucher specimens, post-release genetic surveys of biological control agents also require the use of fine scale molecular markers in order to accurately examine population differentiation. For this, microsatellite markers are often used, but because these markers need to be designed specifically for each study species, the required developmental time can be a barrier to their use. One problem in developing these markers is the time constraints imposed by searching through large numbers of candidate markers (though Next-Generation Sequencing [NGS] has greatly reduced the developmental time over older approaches). I was convinced there was a more efficient approach, and in Chapter 3, I developed a novel

bioinformatics pipeline (Figure 3.1) that searches through NGS data for both the indicative repeat regions that signify microsatellite markers and identifies potentially polymorphic loci. Using this approach I was able to rapidly develop microsatellite markers for two of my study species (*T. pallidus* and *Chromaphis juglandicola*). For both species more than 60% of the target markers amplified and were found to be polymorphic, compared to previous approaches where success rates were much lower (e.g., < 20% [McEwen *et al.* 2011; Jun *et al.* 2012]).

The necessity for post-release genetic surveys is made all the more apparent when unexplained breakdowns in biological control services are observed. Such breakdowns have recently been observed for the walnut aphid (*C. juglandicola*) biological control program in California (Hougardy & Mills 2008). I therefore examined whether population genetic approaches could be used to determine whether two evolutionary factors (hybridization [Mallet 2005] and a geographic mosaic of coevolution [Thompson 1999; Thompson 2005]) may be influencing this system. The effects of hybridization on the sustainability of biological control services are unclear, as studies have found conflicting results (Blair *et al*. 2008; Phillips *et al*. 2008; Szűcs *et al*. 2012; Bean *et al*. 2013; Williams *et al*. 2014) and hybridization has also become a recent concern in regard to native species conservation (Yara *et al.* 2010; Havill *et al.* 2012). No study, however, to my knowledge has used genetic tools to examine the presence of a geographic mosaic of coevolution in a biological control system. In Chapter 4 I found that *T. pallidus* is not monophyletic (Figure 4.1) and appears to be comprised of at least two cryptic species – one of which is a generalist on tree-dwelling panaphine aphids while the other appears to be a specialist on *Myzocallis* species (Figure 4.3). However, contrary to my expectation that hybridization might be responsible for the breakdown of this system, rates of hybridization were too low to be a leading candidate for the recent resumption of in-season insecticide applications for aphid management (Figures 4.4 & 4.5). I did, however, find evidence for the presence of a geographic mosaic of co-evolution (Chapter 5) based on the presence of a selection mosaic as indicated by differing levels of genetic differentiation (Figure 5.1), trait-remixing (Table 5.3), and the presence of coevolutionary hotspots and coldspots (Figure 5.2).

I then compared the results from the walnut aphid system to another system where breakdown in biological control services has occurred, the Argentine stem weevil biological control program in New Zealand (Goldson *et al*. 2014). If, as expected, coevolutionary interactions are important for explaining the overwhelming stability of biological control programs (Holt & Hochberg 1997), and that coevolutionary dynamics can be influenced by metapopulation dynamics (Thompson 1999, 2005), then it is possible that a spectrum of longer-term outcomes might exist. On the one hand, when coevolutionary interactions and metapopulation dynamics are weak, breakdown of a biological control program might develop over a relatively short period of time (as in the Argentine stem weevil system). On the other hand, when coevolutionary interactions and metapopulation dynamics are strong, stable biological control services are likely to be sustained (as expected by Holt & Hochberg 1997). Between these two ends of the spectrum coevolutionary interactions could result in localized breakdowns in biological control services as locations oscillate between coevolutionary hotspots and coldspots due to temporal variation in levels of geneflow. To what extent other biological control programs might occupy a more intermediate position in this spectrum, however, remains unknown due to lack of long-term monitoring and the previous need for *a priori* trait measurement to determine the presence of geographic mosaics of coevolution. With the

availability of a novel population genomics approach to identifying the presence of geographic mosaics of coevolution (Vermeer et al. 2011), greater effort should be directed towards analyzing the coevolutionary interactions of other biological control programs. This would enhance our understanding of whether hotspots or coldspots of coevolution represent the ideal management condition for sustainable biological control services, and how best to manage interacting populations to limit the impact of localized disruptions.

Endosymbionts may also play an important role in the sustainability of biological control services as they may influence host range and rates of hybridization between strains of biological control agents. Therefore, in Chapter 6 I developed molecular markers to conduct post-release genetic surveys of hybridization rates and patterns of endosymbiont inheritance for two strains of the biological control agent *Aphalara itadori* that are currently under review for release in the United States and Canada. The SNP array I developed was able to differentiate between pure and hybrid individuals (Figure 6.1; Appendix Figure 2), and while I found no barriers to introgression of nuclear genomes, I found a curious patterns of endosymbiont inheritance that did not match expectations (Figures 6.1 & 6.2). The result of one of the hybrid crosses showed that offspring had the primary endosymbiont *Candidatus Carsonella rubii* from their paternal strain rather than their maternal strain. In addition, during this cross a low frequency haplotype (~ 8%) of a secondary endosymbiont *Sodalis* sp. found in the paternal strain completely replaced the high frequency haplotype. While it is unclear what mechanisms allowed for the horizontal transmission of the paternal *Carsonella* haplotype and the fixation and/or horizontal transmission of the low frequency *Sodalis* haplotype, previous work (Grevstad *et al.* 2013) showed a severe fitness effect for the psyllid strains when reared on their non-preferred host plants. Therefore, I believe that these endosymbionts may be playing a role in shaping the patterns of host range for each strain of the psyllid and in the fitness of these strains on different knotweed species, though further research will be required to confirm this finding and to identify the mechanism of horizontal transmission in this system.

In conclusion, population genetic approaches provide valuable tools for the study of post-release dynamics in biological control systems. While biological control programs promise to be useful study systems of evolution in action (Roderick & Navajas 2003; Roderick *et al*. 2012), post-release studies will allow for that promise to come to fruition. In my future research endeavors I would like to continue to monitor the effects of hybridization and explore the frequency of geographic mosaics of coevolution in biological control systems. In addition, I would like to conduct post-release population genetic studies of both successful introductions and programs that resulted in failure to establish and/or to control the target pest. I believe these post-release studies will allow us to better determine the evolutionary factors that affect the sustainability of biological control services.

Chapter 7. References

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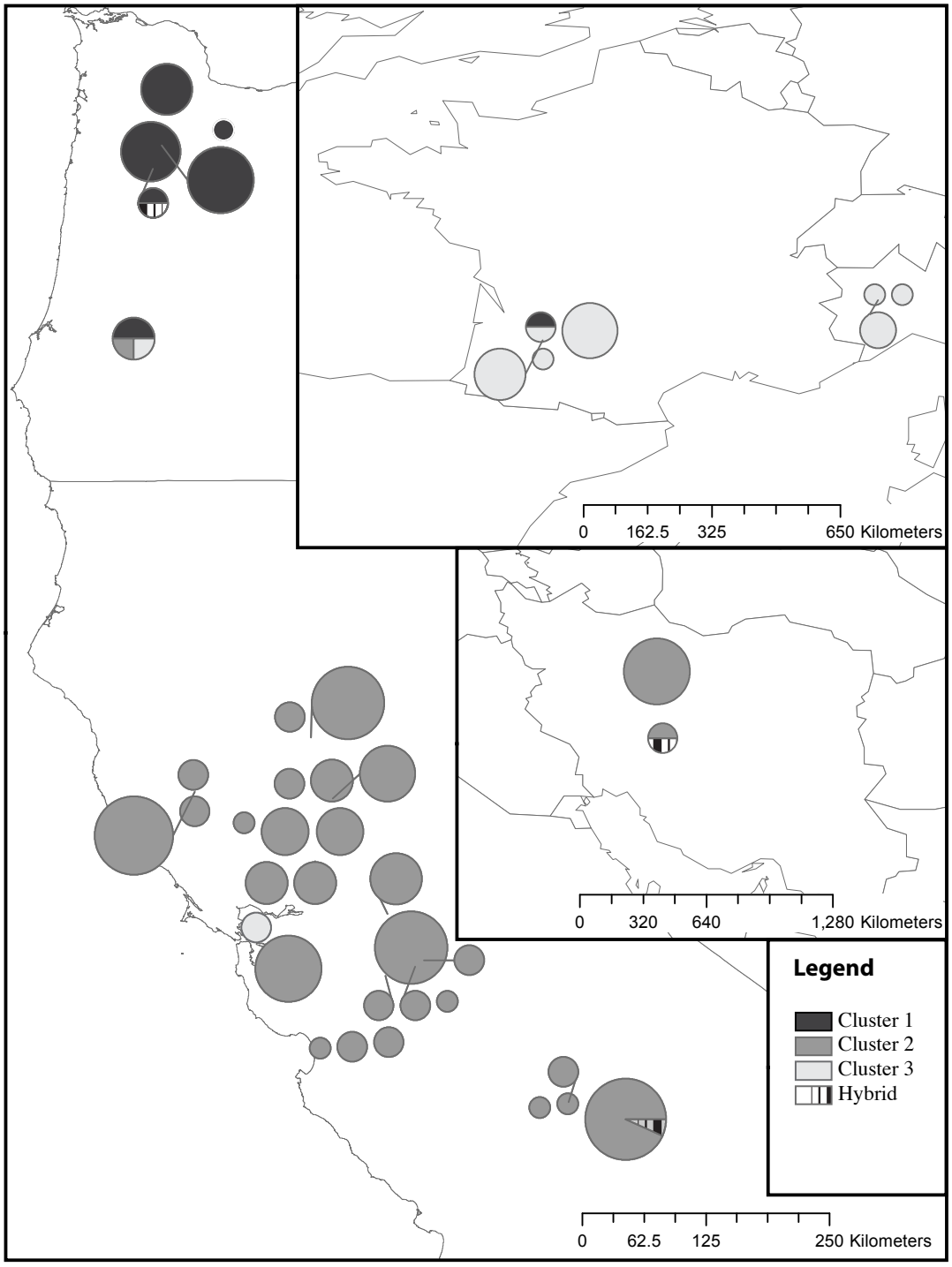
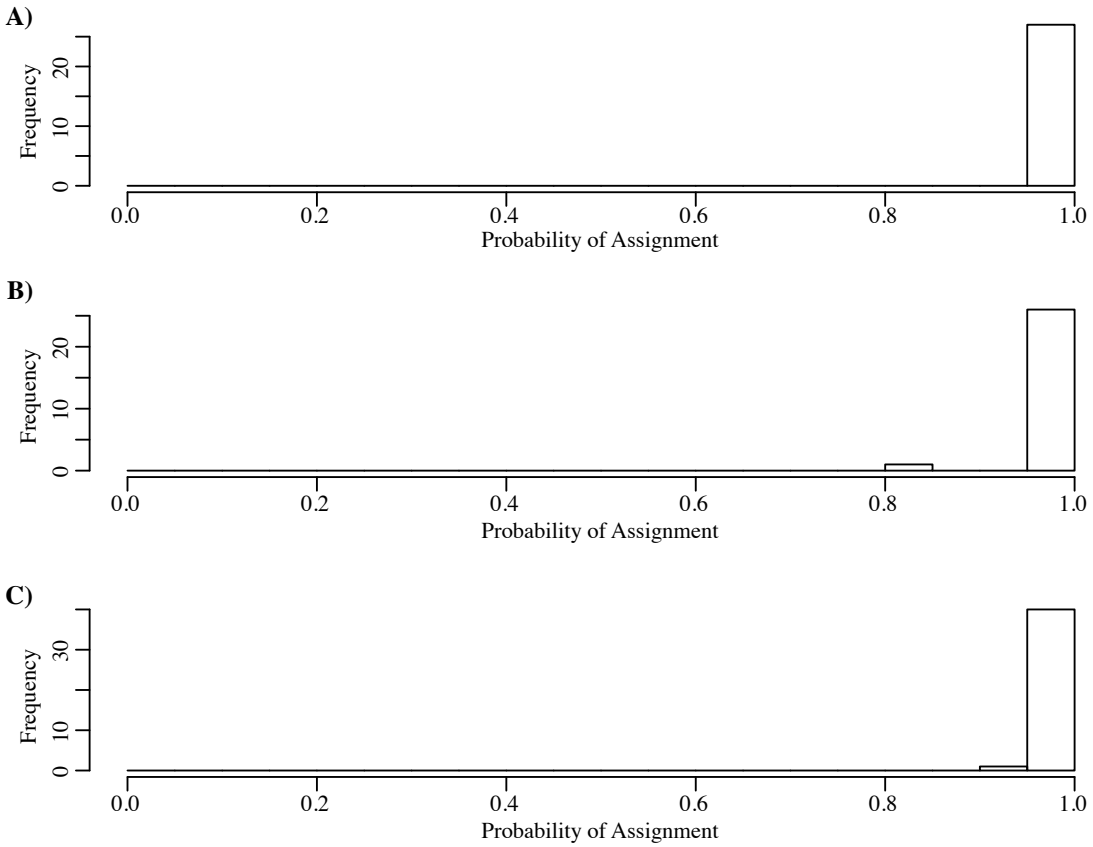


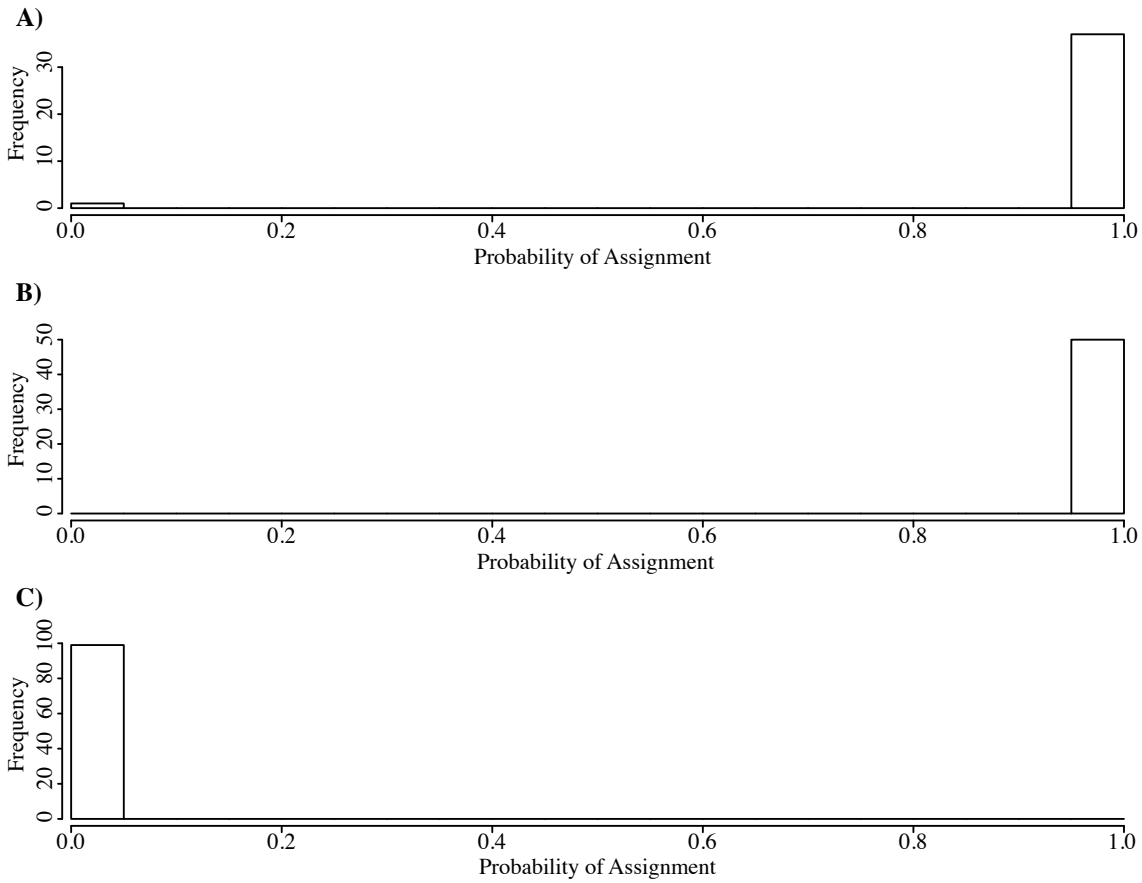
Appendix Figure 1

Sample sites, including the number of individuals and the genetic cluster to which each individual was assigned as per Figure 4.6. The size of each circle is representative of the number of individuals sampled at that location. One location, J0189, is not drawn as individuals from this location emerged in quarantine at the University of California Berkeley but are derived from material collected in Italy from *Myzocallis coryli.*



Appendix Figure 2

Frequency distributions for the probability of assignment scores from the full array for A) Hokkaido, B) Kyushu and C) hybrid individuals to their respective classes using NewHybrids.



Appendix Figure 3

Frequency distributions for the probability of assignment scores from the reduced array for A) Hokkaido, B) Kyushu and C) hybrid individuals to their respective classes using NewHybrids.

Appendix Table 1

Single best match identification for each contig using the blast-n algorithm against the NCBI GenBank Database. The genomic origin of each contig is recorded as insect, microbial, virus, or "other" for all contigs that received an E value < 0.05. Available online at: https://drive.google.com/file/d/0B7A_5EGUmGHBQi1lMHhMV1VSUms/view?usp=sharing

Appendix Table 2

A list of SNP markers used for genotyping, the contig from which they were isolate, their location on the contig, their quality scores, possible allele states, and FASTA sequence including 300 bp of 5' and 3' sequence. Available online at: https://drive.google.com/file/d/0B7A_5EGUmGHBUm5BSkJBYVFES1E/view?usp=sharing

Appendix Tables 3 & 4

Results from our SNP genotyping analyses of the full array (first worksheet) and the reduced array (second worksheet) including the proportion of individuals for which each SNP amplified. Available online at: https://docs.google.com/spreadsheets/d/1RG9rBpBAlm9xj0RXGR81S1_w8E6R52iXfgeLSiOUvcM/edit?usp=sharing