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The Evolution of Peristenus (Hymenoptera: Braconidae): Taxonomy, Phylogenetics and Ecological Speciation

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THE EVOLUTION OF *PERISTENUS* (HYMENOPTERA: BRACONIDAE): TAXONOMY, PHYLOGENETICS, AND ECOLOGICAL SPECIATION

by

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ABSTRACT

Parasitoid wasps are ecologically and economically important as biological control agents. However, little is known about the diversity, distribution and biology of most hymenopteran parasitoids due to their small size, morphological conservatism, and complex life styles. The focus of my PhD research was to investigate the evolution and speciation of euphorine braconid wasps, using a combination of multilocus phylogenetics and population genomic techniques combined with traditional taxonomy. The three data chapters of my dissertation are divided into different taxonomic ranks of euphorine braconids, focusing on genera, species, and populations. For chapter 2, I built a multilocus phylogeny of the tribe Euphorini with extensive taxa sampling around the globe. I confirmed the monophyly of *Peristenus* and *Leiophron*, two important biological control agents, and provided updated generic concepts and identification resources to aid applied researchers. In Chapters 3 and 4, I focused on cryptic species within the *Peristenus pallipes* complex in North America. I used an integrative taxonomic approach to resolve the taxonomic confusion within the Nearctic *Peristenus pallipes* complex (Chapter 3), then I used ddRADSeq to examine their evolutionary relationships with their *Lygus* hosts (Chapter 4). My dissertation provided a comprehensive analysis of *Peristenus* at multiple taxonomic ranks using phylogenetics and population genomics, providing insights into their evolutionary history that can be extrapolated into other groups of parasitoid wasps. The results from these studies also advanced our understanding of this group of animals of theoretical, economical, and conservation importance.

Keywords: Parasitoid, Phylogenetics, Evolution, Braconidae, Speciation, ddRADSeq

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CHAPTER ONE: INTRODUCTION

Understanding the origins, drivers, and maintenance of biodiversity at different evolutionary scales is a central goal of contemporary biology. These scales range from the origin of new species and the role that species interactions play in lineage proliferation, to macroevolutionary patterns that reveal the extent to which species interactions influence diversification across increasing temporal and geographic scales (Cutter 2013; Johnson & Stinchcombe 2007; Schluter 2001; Thompson 2005). With an estimated 5.5 million living species, insects are the most diverse multicellular organisms on Earth, making them a particularly interesting group for the study of animal diversity (Grimaldi & Engel 2005; Stork 2018). Herbivorous insects and their insect predators are especially species-rich, forming complex interactive webs across multiple trophic levels (Mayhew 2007; Ødegaard 2000). The most diverse and complex insect systems – those most promising for seeking understanding regarding the origins of diversity – are often also the most poorly known, such that an in-depth study of evolutionary dynamics requires a parallel effort to discover and describe new diversity.

The largest orders of insects are the Coleoptera (beetles), Diptera (flies), Lepidoptera (moths and butterflies), and Hymenoptera (sawflies, ants, bees and wasps). Hymenoptera is a large order of an estimated 153,000 species and possibly up to one million undescribed extant species (Grimaldi & Engel 2005; Peters et al. 2017). As parasitoids, predators, and pollinators, Hymenoptera play a fundamental role in terrestrial ecosystems and are of substantial economic importance (Grimaldi & Engel 2005; Peters et al. 2017).

The majority of Hymenoptera are parasitoids, a specialized insect that feeds on a single arthropod host which it ultimately kills (Godfray 1994). Despite having only evolved once within Hymenoptera, parasitism has led to an explosive radiation that is often contributed as one of the

most successful adaptations in insects (Grimaldi & Engel 2005). As a result of their diverse host choice, parasitoids are regulators of arthropod populations, and provide important ecological and economic services as biological control agents (Heraty et al. 2011). Many cryptic species complexes exist within Hymenoptera, which results in high levels of misidentifications in the literature. These factors subsequently renders many host records and distributions of parasitoids unreliable, and present challenges in accurate identification of Hymenoptera using morphological characters for species delimitation and identification (Santos & Quicke 2011). Fortunately, with recent advances in utilizing molecular data in conjunction with morphological and ecological data, identification of cryptic species with accurate host records is now possible (Fernández-Triana et al. 2014; Smith et al. 2006).

The focus of my PhD research was to investigate the evolution and speciation patterns of euphorine braconid wasps, using a combination of molecular phylogenetics, population genomics, and traditional morphological taxonomy. The three data chapters of my dissertation examined different taxonomic ranks of euphorine braconids, focusing on genera, species, and populations. For **Chapter 2**, I used multiple genes and samples collected from around the world to resolve the phylogenetic relationships of euphorine wasps in the tribe Euphorini. I also updated the generic concepts of *Peristenus* and *Leiophron* with new distinguishing characters, clarifying the taxonomic confusion with the classification among genera. **Chapter 3** focused on resolving morphologically cryptic species in the *Peristenus pallipes* complex in North America using a combination of morphological differences, DNA, host records, and species distributions. Nine previously named species were synonymized to three as a result of this study, demonstrating that traditional techniques that focused solely on morphology are unreliable in identifying these economically important parasitoid wasps. The importance of native species loss

due to competition with introduced biocontrol agents were highlighted using *P. pallipes* complex as an example. In addition, I urged collaborations between taxonomists, ecologists, and applied entomologists to ensure the accurate selection of biocontrol agents, and long term studies post release to ensure the impact of foreign agents with native species. Finally, as a follow up study to chapter 3, I also used genomic data to understand the drivers of *Peristenus* speciation. In **Chapter 4,** I examined populations of two *Peristenus* species with overlapping ranges and their hosts using thousands of genome-wide differences known as single nucleotide polymorphisms. *Peristenus* were sampled from different locations, times, host plants, and host insects. While the insect hosts did not exhibit genetic differences between different host plants, the two *Peristenus* species were separated by differences in emergence time. This temporal difference could suggest that natural selection can act on adult emergence time to avoid competition with another species. By integrating state-of-the-art genomic tools with traditional morphological and ecological data, I was able to accurate identify and untangle evolutionary relationships among taxonomically challenging groups. This has a direct effect on the conservation of these often-overlooked but ecological and economically important insects, as well as reveal insights into the radiation of one of the most diverse lineages of animals on this planet.

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CHAPTER TWO: MULTILOCUS PHYLOGENY OF THE PARASITIC WASPS IN THE TRIBE EUPHORINI (HYMENOPTERA: BRACONIDAE) WITH REVISED GENERIC CLASSIFICATIONS

This chapter has been accepted for publication to PeerJ: Zhang, Y.M., Stigenberg, J., Meyer, J.H., and Sharanowski, B.J. Multilocus Phylogeny of the Parasitic Wasps in the Tribe Euphorini (Hymenoptera: Braconidae) with Revised Generic Classifications.

Abstract

Parasitic wasps in the family Braconidae are important regulators of insect pests, particularly in forest and agroecosystems. Within Braconidae, wasps in the tribe Euphorini (Euphorinae) attack economically damaging plant bugs (Miridae) that are major pests of field and vegetable crops. However, the evolutionary relationships of this tribe have been historically problematic. Most generic concepts have been based on ambiguous morphological characters which often leads to misidentification, complicating their use in biological control. Using a combination of 3 genes (*COI*, *28S*, and *CAD*) and 81 taxa collected worldwide, the monophyly of the tribe Euphorini and the two genera *Peristenus* and *Leiophron* were confirmed using maximum likelihood and Bayesian inference. The subgeneric classifications of *Leiophron sensu lato* were not supported, therefore *Euphoriella*, *Euphoriana*, and *Euphorus* have been synonymized under *Leiophron*. The monotypic genus *Mama* was not supported and thus, *Mama mariae* **syn. n** was placed as a junior synonym of *Leiophron reclinator*. The generic concepts of *Peristenus* and *Leiophron* were refined to reflect the updated phylogeny. Further we discuss the need for revising Euphorini given the number of undescribed species within the tribe.

Introduction

Braconid wasps in the large and diverse subfamily Euphorinae is divided into 14 tribes and 52 genera (Stigenberg et al. 2015). Euphorines attack a variety of host life stages ranging from nymphal/larval hosts to adults of seven different orders of insects: Coleoptera, Hemiptera, Hymenoptera, Neuroptera, Orthoptera, Psocodea, and Lepidoptera (Chen & van Achterberg, 1997; Shaw, 1988; Stigenberg et al. 2015). The tribe Euphorini Förster contains koinobiont endoparasitoids of Hemiptera and Psocodea, which attack young nymphs (1st or 2nd instar) and feed internally on the hemolymph of their hosts (Loan, 1974a). Mature parasitoid larvae emerge from mature host nymphs or teneral adults, and overwinter as pupae in soil (Loan, 1974a). Several species of Euphorini have been extensively studied for their use in biological control programs because they attack many serious agricultural pests such as *Lygus* Hahn (e.g. Day, 1987; Haye et al. 2005; Haye et al. 2007). Despite the research interest using Euphorini wasps in applied entomology, the classification and identification of these parasitoids remains challenging, largely due to the ambiguous generic concepts leading to taxonomic uncertainty and misidentification.

The taxonomic history of Euphorini is a long and convoluted one. *Euphoriana* Gahan, *Euphoriella* Ashmead, *Euphorus* Nees, and *Peristenus* Förster have all been synonymized under or treated as subgenera of *Leiophron* in a variety of combinations by different authors (Chen & van Achterberg 1997; Loan 1974b; Stigenberg et al. 2015). In addition, *Aridelus* Marshall, *Chrysopophthorus* Goidanich, *Cryptoxilos* Viereck, *Holdawayella* Loan, *Mama* Belokobylskij, and *Wesmaelia* Förster were also included in Euphorini until the most recent comprehensive revision of the entire subfamily Euphorinae by Stigenberg et al. (2015). Only a few exemplars of

Euphorini were included in (Stigenberg et al. 2015), but the monophyly of the tribe was strongly supported. Currently there are three recognized genera within Euphorini, including *Leiophron sensu lato*, *Peristenus*, and the monotypic *Mama* (Stigenberg et al. 2015). *Leiophron* is further divided into four subgenera, *Euphoriana*, *Euphoriella*, *Euphorus*, and *Leiophron sensu stricto* (Stigenberg et al. 2015).

Here, we extensively sample Euphorini wasps and reconstruct the evolutionary relationships among its members using a multi-locus dataset. We reassess the generic and subgeneric concepts of Euphorini and revise the classification to reflect the phylogeny. The results of this study provide a comprehensive framework for phylogenetic relationships among Euphorini wasps and we provide taxonomic clarity and identification resources to aid future applied research and biological control programs.

Materials and Methods

Sample Collection

Specimens were borrowed from the following institutions and curators: Hymenoptera Institute Collection, University of Kentucky (HIC, M. Sharkey), French National Museum of Natural History (MNHN, C. Villemant), Swedish Museum of Natural History (NHRS, H. Vårdal), and Zoological Institute of Russian Academy of Sciences (ZIN, S. Belokobylskij). Additional specimens were collected via sweep netting or Malaise trap samples from Canada, USA, and Peru. Specimens were identified using Chen & van Achterberg 1997, Loan 1974a, 1974b, and Stigenberg & van Achterberg (2016). Outgroups included representatives of the most closely related tribes to Euphorini, based on Stigenberg et al. (2015): *Microctonus* Wesmael

(Perilitini), *Townesilitus* Haeselbarth & Loan (Townsilitini), and *Chrysopophthorus* Goidanich (Helorimorphini). A list of the specimens utilized in this study is provided in Table 2.1, and detailed locality information in Table A2.1. A map of the distribution of these specimens is depicted in Figure 2.1, which was generated using ArcMap v10.5.1. For ease of interpretation of results, specimen information was added to taxon labels for the phylogenetic analyses, including country and lowest identification. The subgeneric names within *Leiophron s.l.* are used as specimen names to avoid confusion with *Leiophron s.s.* (eg. *Leiophron (Leiophron) uniformis* is listed as *Leiophron uniformis*, whereas *Leiophron (Euphoriana) dispar* islisted as *Euphoriana dispar*).

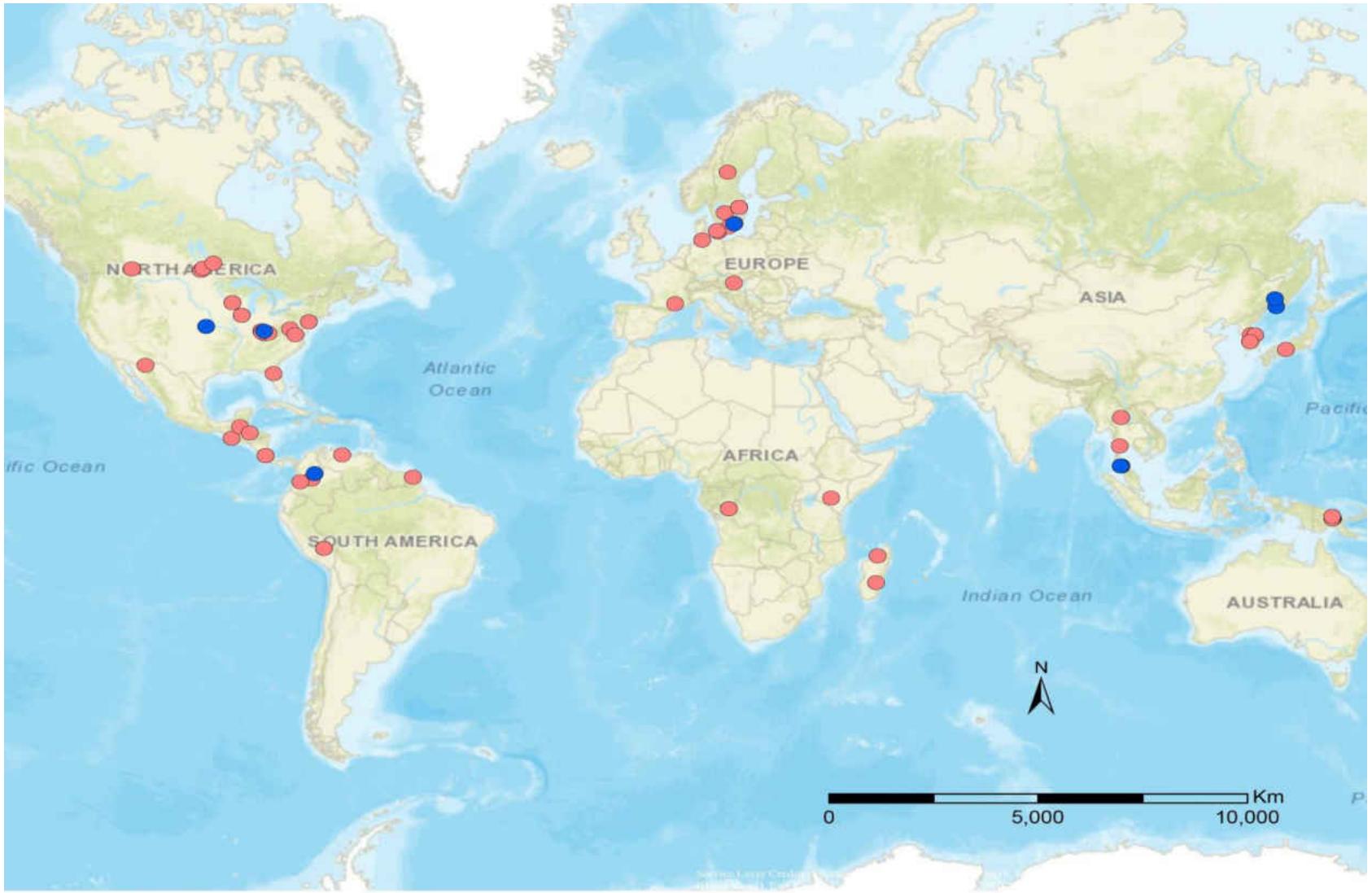

Figure 2.1. Geographical distribution of specimens used in this study. Blue dots are published data from Stigenberg et al. 2015, red dots are newly sampled taxa for this study.

Table 2.1. GenBank accession numbers (new sequences generated for this study is in bold), collection localities, and voucher deposition institutes for all specimens used in this study. HIC (Hymenoptera Institute, University of Kentucky, Lexington), MNHN (French National Museum of Natural History, Paris), NHRS (Swedish Museum of Natural History, Stockholm), UCFC (University of Central Florida Collection of Arthropods, Orlando), ZIN (Zoological Institute of Russian Academy of Sciences, St. Petersburg).

Taxon Label	***COI***	***28S***	***CAD***	**Locality**	**Voucher Location**
07_Yves_Leiophron_PNG	MG926854	MG913702	-	Papua New Guinea	MNHN
08_Yves_Leiophron_PNG	MG926855	MG913703	-	Papua New Guinea	MNHN
10_Yves_Leiophron_PNG	MG926856	MG913704	-	Papua New Guinea	MNHN
AB016_Peristenus_KS	KJ591487	KJ591282	-	USA	HIC
AB020_Peristenus_KY	KJ591488	KJ591283	-	USA	HIC
AB023_Peristenus_KS	KJ591489	KJ591284	-	USA	HIC
Euph_001_Euphorus_pallidistigma_SWE	MG926857	-	MG913762	Sweden	NHRS
Euph_017_Peristenus_JAP	MG926858	-	MG913763	Japan	NHRS
Euph_020_Peristenus_SWE	MG926859	MG913705	MG913764	Sweden	NHRS
Euph_083_Peristenus_HUN	MG926860	MG913706	-	Hungary	NHRS
Euph_162_Leiophron_apicalis_SWE	MG926861	-	-	Sweden	NHRS
JS01000238_Leiophron_fascipennis_SWE	MG926862	MG913713	-	Sweden	NHRS

Taxon Label	***COI***	***28S***	***CAD***	**Locality**	**Voucher Location**
JS01000242_Leiophron_SWE	KJ591452	KJ591243	-	Sweden	NHRS
JS01000267_Leiophron_FRGU	MG926863	MG913707	-	French Guiana	NHRS
JS01000499_Mama_mariae_RUS	KJ591460	KJ591250	-	Russia	ZIN
JS01000515_Euphoriana_dispar_RUS	KJ591458	MG913708	-	Russia	ZIN
JS01000538_Euphorus_duploclaviventris_SWE	MG926864	-	-	Sweden	NHRS
JS01000539_Euphorus_oblitus_SWE	MG926865	-	-	Sweden	NHRS
JS01000540_Leiophron_deficiens_SWE	MG926866	MG913709	-	Sweden	NHRS
JS01000542_Leiophron_reclinator_SWE	MG926867	-	-	Sweden	NHRS
JS01000547_ Leiophron _MAD	MG926868	MG913710	-	Madagascar	NHRS
JS01000552_Peristenus_SWE	MG926869	MG913711	MG913765	Sweden	NHRS
JS01000553_Euphorus_basalis_SWE	MG926870	-	MG913766	Sweden	NHRS
JS01000554_Euphorus_fulvipes_SWE	MG926871	MG913712	MG913767	Sweden	NHRS
JS068_Leiophron_COL	KJ591455	KJ591246	KJ591362	Colombia	HIC
JS120_Leiophron_THA	KJ591456	KJ591247	KJ591363	Thailand	HIC
JS129_Leiophron_THA	KJ591457	KJ591248	KJ591364	Thailand	HIC
PNG_5_Leiophron	MG926872	MG913714	-	Papua New Guinea	MNHN

Taxon Label	***COI***	***28S***	***CAD***	**Locality**	**Voucher Location**
PNG_6_Leiophron	MG926873	MG913715	-	Papua New Guinea	MNHN
PNG_7_Leiophron	MG926874	MG913716	-	Papua New Guinea	MNHN
YMZ038_Peristenus_GER	MG926875	MG913717	MG913768	Germany	UCFC
YMZ077_Leiophron_uniformis_MB	-	MG913718	MG913769	Canada	UCFC
YMZ081_Euphoriella_MB	MG926876	MG913719	MG913770	Canada	UCFC
YMZ124_Peristenus_mellipes_MB	KY566090	MG913720	MG913771	Canada	UCFC
YMZ132_Leiophron_KY	MG926877	MG913721	MG913772	USA	UCFC
YMZ133_Leiophron_KY	MG926878	MG913722	MG913773	USA	UCFC
YMZ134_Leiophron_WV	MG926879	MG913723	MG913774	USA	UCFC
YMZ136_Leiophron_KY	MG926880	MG913724	MG913775	USA	UCFC
YMZ139_Leiophron_uniformis_FRA	MG926881	MG913725	-	France	UCFC
YMZ141_Leiophron_THA	MG926882	MG913726	MG913776	Thailand	UCFC
YMZ142_Peristenus_MAD	MG926883	MG913727	-	Madagascar	UCFC
YMZ145_Leiophron_COL	MG926884	MG913728	MG913777	Colombia	UCFC
YMZ146_Leiophron_COL	MG926885	MG913729	-	Colombia	UCFC
YMZ148_Euphoriella_GUA	MG926886	MG913730	MG913778	Guatemala	UCFC

Taxon Label	***COI***	***28S***	***CAD***	**Locality**	**Voucher Location**
YMZ211_Peristenus_dayi_MB	KY566098	MG913731	MG913779	Canada	UCFC
YMZ335_Peristenus_howardi_AB	KY566100	MG913732	MG913780	Canada	UCFC
YMZ341_Peristenus_relictus	KY566106	MG913733	MG913781	USA	UCFC
YMZ343_Peristenus_digoneuti	MG926887	MG913734	MG913782	USA	UCFC
YMZ345_Leiophron_KY	MG926888	MG913735	-	USA	UCFC
YMZ346_Peristenus_WI	MG926889	MG913736	-	USA	UCFC
YMZ349_Peristenus_IL	MG926891	MG913738	MG913783	USA	UCFC
YMZ351_Leiophron_VA	MG926892	MG913739	-	USA	UCFC
YMZ356_Peristenus_WI	MG926893	MG913740	-	USA	UCFC
YMZ358_Euphoriella_KY	MG926894	MG913741	-	USA	UCFC
YMZ359_Euphoriella_FL	MG926895	MG913742	-	USA	UCFC
YMZ361_Leiophron_AZ	MG926896	MG913743	MG913784	USA	UCFC
YMZ363_Euphoriella_COL	MG926897	MG913744	-	Colombia	UCFC
YMZ364_ Leiophron _CR	MG926898	MG913745	-	Costa Rica	UCFC
YMZ365_Euphoriella_CR	MG926899	MG913746	MG913785	Costa Rica	UCFC
YMZ366_Euphoriella_GUA	MG926900	MG913747	-	Guatemala	UCFC

Taxon Label	***COI***	***28S***	***CAD***	**Locality**	**Voucher Location**
YMZ367_Leiophron_HON	MG926901	MG913748	-	Honduras	UCFC
YMZ368_Leiophron_VEN	MG926902	MG913749	-	Venezuela	UCFC
YMZ370_Euphoriella_PER	MG926903	MG913750	MG913786	Peru	UCFC
YMZ371_Leiophron_PER	MG926904	MG913751	-	Peru	UCFC
YMZ372_Peristenus_PER	MG926905	MG913752	MG913787	Peru	UCFC
YMZ373_Leiophron_THA	MG926906	-		Thailand	UCFC
YMZ375_Leiophron_KEN	MG926907	MG914753	-	Kenya	UCFC
YMZ376_Leiophron_THA	MG926908	MG914754	MG913788	Thailand	UCFC
YMZ377_Leiophron_THA	MG926909	MG914755	MG913789	Thailand	UCFC
YMZ378_Leiophron_THA	MG926910	MG914756	MG913790	Thailand	UCFC
YMZ380_Leiophron_CON	MG926911	-	MG913791	Congo	UCFC
YMZ382_Leiophron_KOR	MG926912	-	MG913792	South Korea	UCFC
YMZ383_Leiophron_KOR	MG926913	MG914757	-	South Korea	UCFC
YMZ384_Leiophron_KOR	MG926914	MG914758	-	South Korea	UCFC
YMZ385_Leiophron_KOR	MG926915	MG914759	-	South Korea	UCFC
YMZ386_Leiophron_CON	MG926916	MG914760	-	Congo	UCFC

Taxon Label	***COI***	***28S***	***CAD***	**Locality**	**Voucher Location**
YMZ388_Leiophron_pallidistigma_KOR	KJ591473	KJ591262	KJ591397	South Korea	UCFC
AB102 Microctonus (Perilitini)	KJ591529	KJ591329	KJ591412	USA	HIC
JS01000218 Townesilitus (Townesilitini)	KJ591440	KJ591228	KJ591353	Sweden	HIC
JS115 Chrysopophthorus (Helorimorphini)	MG926854	MG913702	-	Colombia	HIC

Terminology and Image Capture

Terminology used for most morphological characters follows Chen & van Achterberg (1997) and Stigenberg et al. (2015). However, wing venation terminology follows Sharkey & Wharton (1997). Specimens were photographed using a Canon 7D Mark II with a Mitutoyo M Plan Apo 10× objective mounted onto the Canon EF Telephoto 70–200mm zoom lens, and the Canon MT–24EX Macro Twin Lite Flash (Tokyo, Japan) with custom-made diffusers to minimize hot spots.

DNA Protocols

A total of 80 taxa were sampled out of which three species represented outgroups. The taxon sampling covers the entire range of Euphorini, which is found on all continents except in Antarctica and Australia outside of Papua New Guinea (Yu et al. 2012). This is the largest sampling of any of the euphorine tribes, and is comprised of all three Euphorini genera, as well as all four subgenera of *Leiophron s.l.* Specimens were extracted, amplified, and sequenced either at the Molecular Systematics Laboratory, Swedish Museum of Natural History following the protocols listed in Stigenberg et al. (2015), or at the Insect Systematics Laboratory at the University of Central Florida following the DNeasy™ Tissue Kit protocol (Qiagen, Valencia, CA, U.S.A.). Petioles were separated from mesosomas to ensure buffer penetration during tissue lysis, and the two body parts were mounted onto the same point post-extraction for vouchering. Voucher specimens are listed in Table 2.1. Three genes were amplified: partial *28S* domains 2 and 3 (rDNA), partial *CAD* (Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, And Dihydroorotase) and the 5’ region of mitochondrial *COI*. New Euphorini-specific primers were designed for *CAD* based on sequences from Sharanowski et al. (2011) and Stigenberg et al.

(2015). The faster rate of evolution of the mitochondrial genes is ideal for separating closely related species (Zhang et al. 2017), while the ribosomal and nuclear genes have slower rates of evolution and are more suitable for higher level phylogenetic relationships (Sharanowski et al. 2011). All three genes are commonly used in Braconidae phylogenetics, including Euphorinae (Sharanowski et al. 2011; Stigenberg et al. 2015; Zhang et al. 2017).

All PCRs were performed on a Bio-Rad MyCyclerTM thermal cycler, using approximately 1µg DNA extract, 1X Standard Taq Buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, pH 8.3, New England Biolabs, Ipswich, Massachusetts, U.S.A.), 200 µM dNTP (Invitrogen, Carlsbad, California, U.S.A.), 4 mM $MgSO_4$, 400 nM of each primer, 1 unit of Taq DNA polymerase (New England Biolabs), and purified water to a final volume of 25 µl. Primer information and PCR conditions are listed in Table 2.2. Amplicons of reaction products were cleaned with Agencourt CleanSEQ magnetic beads and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) and the Applied Biosystems 3730xl DNA Analyzer at the University of Kentucky, Advanced Genetic Technologies Center (UK-AGTC). Contigs were assembled and edited using Geneious version 8.18 (Kearse et al. 2012), and alignment was conducted using MAFFT server (Katoh et al. 2002; https://mafft.cbrc.jp/alignment/server/). The protein coding genes were aligned using default MAFFT settings, and for *28S* we used Q-INS-I strategy (Katoh & Toh, 2008) which takes secondary RNA structure into account. New sequences obtained from this study were deposited in GenBank (See Table 2.1).

Table 2.2. List of primers used in this study.

Gene region	Primers	Sequence (5' to 3')	Source	Annealing Temperature
28S	D2F (fwd)	AGTCGTGTTGCTTGATAGTGCAG	Campbell et al. (1993)	55ºC
	D2R (rev)	TTGGTCCGTGTTTCAAGACGGG	Campbell et al. (1993)	55ºC
COI	LCO1490 (fwd)	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	49ºC
	HCO2198 (rev)	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)	49ºC
CAD	CAD2F (fwd)	TAYGAGCTTACCAAAATWGAYC	New primer	52ºC
	CAD2R (rev)	CATAAATGTCCATCACAACTTC	New primer	52ºC

Phylogenetic Analyses

The three genes were analyzed separately and concatenated using Bayesian inference (BI) analysis with MrBayes v3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway (Miller et al. 2009). Each analysis had two independent searches with four chains and were run for 10,000,000 generations, sampling every 1000, with a 10% burnin discarded. For the concatenated analysis, partitions were separated by gene and codon position for protein-coding genes for a total of six preselected partitions. Six partitions (*28S*; *CAD_1*; *CAD_2 +CAD_3*; *COI_1*; *COI_2*; and *COI_3*) were chosen using PartitionFinder 2.1.1 (Lanfear et al. 2016), based on the greedy algorithm and Bayesian Information Criterion (Table 2.3). The same partitions were also used for a maximum likelihood (ML) analysis using the relatively new IQ-Tree method (Nguyen et al. 2014), with 1000 ultrafast bootstraps developed by Hoang et al. (2017). The concatenated dataset was also analyzed with RAxML v8.2.0 (Stamatakis, 2006), using the GTR+ Γ model of nucleotide substitution and 1000 nonparametric bootstraps. All resulting trees were visualized using FigTree v1.4.2 (Rambaut, 2012). Intraspecific distances between *Mama*

mariae Belokobylskij and *Leiophron reclinator* (Ruthe) was calculated using MEGA v7.0.21 (Kumar et al. 2016) using the Kimura-2-parameter (K2P) model (Kimura, 1980).

Table 2.3. Markers, partitions schemes, and substitution models (Model) according to PartitionFinder2. Additional summary includes number of basepairs (#bp), number of variable sites (#var), number of parsimonious informative sites (#par), and the CG content (CG%).

Marker and partitions	#bp	#var	#par	CG%	Model	References
28S ribosomal DNA						
28S_123	592	263	179	43.3	GTR+G	(Tavaré 1986)
CAD nuclear DNA	507	185	121	36.0		
CAD_1	169	133	96	26.5	HKY+I+G	(Hasegawa et al. 1985)
CAD_23	338	52	25	40.3	HKY+I+G	(Hasegawa et al. 1985)
COI mtDNA	660	360	296	28.4		
COI_1	220	103	78	33.8	GTR+I+G	(Tavaré 1986)
COI_2	220	55	33	38.4	GTR+G	(Tavaré 1986)
COI_3	220	202	185	12.8	GTR+I+G	(Tavaré 1986)

Results

Here we present the most taxonomically comprehensive phylogeny of the euphorine braconid tribe Euphorini with all known genera and subgenera sampled. All genera and subgenera had multiple representatives except *Euphoriana* (only one exemplar included - *E. dispar*) and the monotypic *Mama mariae*. A total of 39 *CAD*, 71 *28S*, and 80 *COI* for a total of

190 sequences were used for the final analyses, 158 of which were newly generated for this study (Table 2.1). The summary statistics of all three genes can be found in Table 2.3. While we failed to amplify *CAD* sequences from some older specimens, the gene itself is informative (see Table 2.2, 2.3) and should be used in other multilocus analyses of braconids. All the individual BI gene trees (Figs. A2.1–A2.3), as well as the concatenated BI and ML (Figs. A2.4 – A2.6) analyses strongly supported the monophyly of the tribe Euphorini (1, 100, 100, for MrBayes posterior probability, RAxML bootstrap support, and IQ-Tree ultrafast bootstrap support, respectively) as well as the monophyly of the genera *Peristenus* (1, 95, 99) and *Leiophron s.l.* (1, 90, 90) (Fig. 2.2).

Mama mariae, the only species from the monotypic genus *Mama*, was not supported as a distinct genus and was instead recovered as a sister group of *Leiophron reclinator* (Fig. 2.2) with 0.8% difference based on *COI* genetic distance.

The four subgenera of *Leiophron s.l.* (*Leiophron s.s.*, *Euphoriana*, *Euphoriella*, *Euphorus*) were not supported as distinct clades within the monophyletic *Leiophron s.l.* in any of the phylogenetic analyses (Figs. 2.2, A2.1 – A2.6).

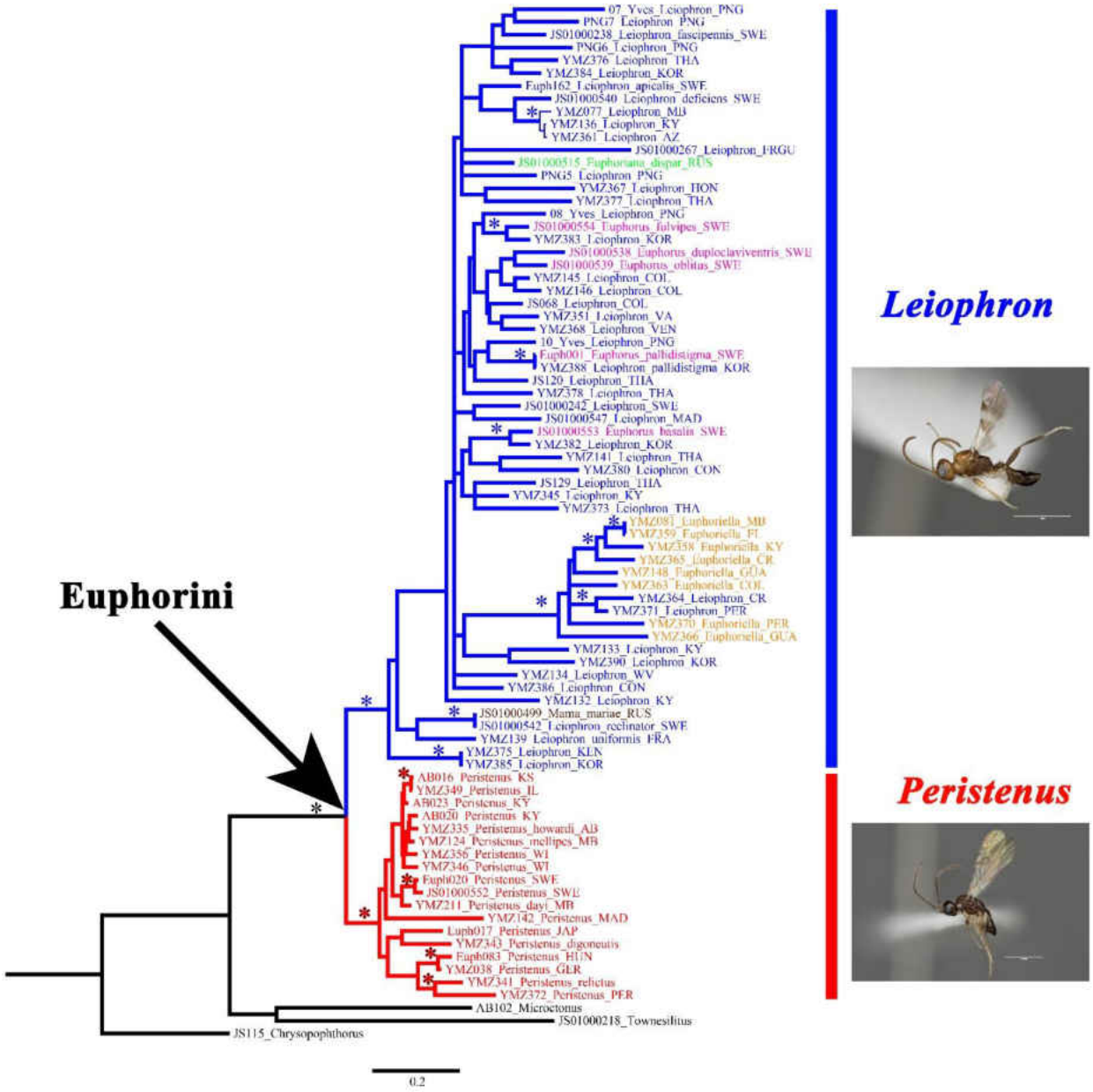

Figure 2.2. Concatenated gene tree for MrBayes, RAxML, and IQ-Tree. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support for all three analyses (≥ 0.98 posterior probability support for MrBayes; ≥90 for bootstrap support for RAxML; and ≥90 for ultrafast bootstrap support for IQ-Tree).

Discussion

Generic Concepts of Peristenus and Leiophron s.l.

Our data corroborates the results of Stigenberg et al. (2015) in supporting the monophyly of *Peristenus* and *Leiophron*, and with a much more focused taxon sampling we were able to delineate the finer relationships within the tribe Euphorini. *Peristenus* is largely uniform in morphology and exclusively attacks Miridae, while its sister taxon *Leiophron* is much more variable in both morphology and host breadth which likely has lead to convergent morphology, and hence the subgeneric concepts and taxonomic confusion. *Peristenus* can be distinguished from *Leiophron* by the evenly setose 1st discal, basal, and subbasal cells in the forewing (Fig. 2.3A), and the 1st metasomal tergite, which is fused or touching basally (Fig. 2.4B).

Representatives of all four subgenera of *Leiophron s.l.* defined by Stigenberg et al. (2015): *Euphoriana*, *Euphoriella*, *Euphorus*, and *Leiophron s.s.*, were included in this analysis. These subgeneric relationships were not supported in any of our analyses, as they failed to form monophyletic clades (Fig. 2.2). This is not surprising given the lack of consistent morphological characters that were used to distinguish them in the past, as specimens often exhibit characteristics of two different subgenera (Stigenberg et al. 2015). Morphological characters such as the presence or absence of the forewing vein (RS + M)a, hindwing vein cu-a, and complete occipital carina are all too variable to be used as defining characteristics (Chen & van Achterberg, 1997; Stigenberg et al. 2015). These ambiguous distinguishing characters at the subgeneric level can easily lead to misidentification in ecological or applied studies. Therefore, based on our molecular evidence combined with the inconsistency of previously used morphological characters, we recommend treating *Leiophron* as a single genus without further

subdivisions and synonymize the subgenera *Euphoriana*, *Euphoriella*, and *Euphorus* as junior synonyms of *Leiophron*. With this taxonomic update, *Leiophron* can be identified with the following combination of characters: 1st discal cell of the forewing is often more setose than basal and subbasal cells in *Leiophron* (Fig. 2.3B), but if not, then the ventral side of the 1st metasomal tergite (petiole) is not fused (Fig. 2.4C).

The exact age of this split between *Peristenus* and *Leiophron* is unknown, as the only known fossil record of Euphorini is a single specimen described as *Euphorus indurescens* Brues, found in Florissant, Colorado and dating back to Eocene at around 33.7 – 37mya (Brues, 1910). Both genera have received little taxonomic attention outside of Europe, North America, and Asia (Belokobylskij 2000b; Chen & van Achterberg 1997; Goulet & Mason 2006; Loan 1974a, 1974b; Mohammad et al. 2009; Stigenberg & van Achterberg 2016). We have included many undescribed species from Central and South America, Africa, and Papua New Guinea, which is unsurprising given the tremendous diversity of their major host Miridae. The first and second author are currently working on describing species from Papua New Guinea (Stigenberg & Zhang, unpublished data), but a revision of the world Euphorini is needed.

Validity of the genus Mama

The validity of the enigmatic genus *Mama*, described based on a single species *M. mariae* from eastern Russia (Belokobylskij, 2000a), has been questioned before by Simbolotti et al. (2004) as both *M. mariae* and *L. reclinator* have long, compressed, and spiny scapes (Fig. 2.4A). Simbolotti et al. (2004) compared the type specimens of *M. mariae* to the morphologically similar *L. reclinator*, but due to the poor condition of the lectotype no definitive conclusion was made. With the consistent placement of the two species as sister taxa with short branch lengths in

all three genes (Figs. A2.1 – A2.3) and concatenated dataset (Fig. 2.2), and the similarity in morphology (the second author has examined the holotypes of *M. mariae* and *L. reclinator*), we synonymize *M. mariae* **syn. n**. as a junior synonym of *L. reclinator*, thus effectively dissolving the monotypic genus *Mama* **syn. n.**. The distribution of *L. reclinator* likely spans across Eurasia, as specimens are found from eastern Russia to Sweden and the United Kingdom (Stigenberg & van Achterberg 2016).

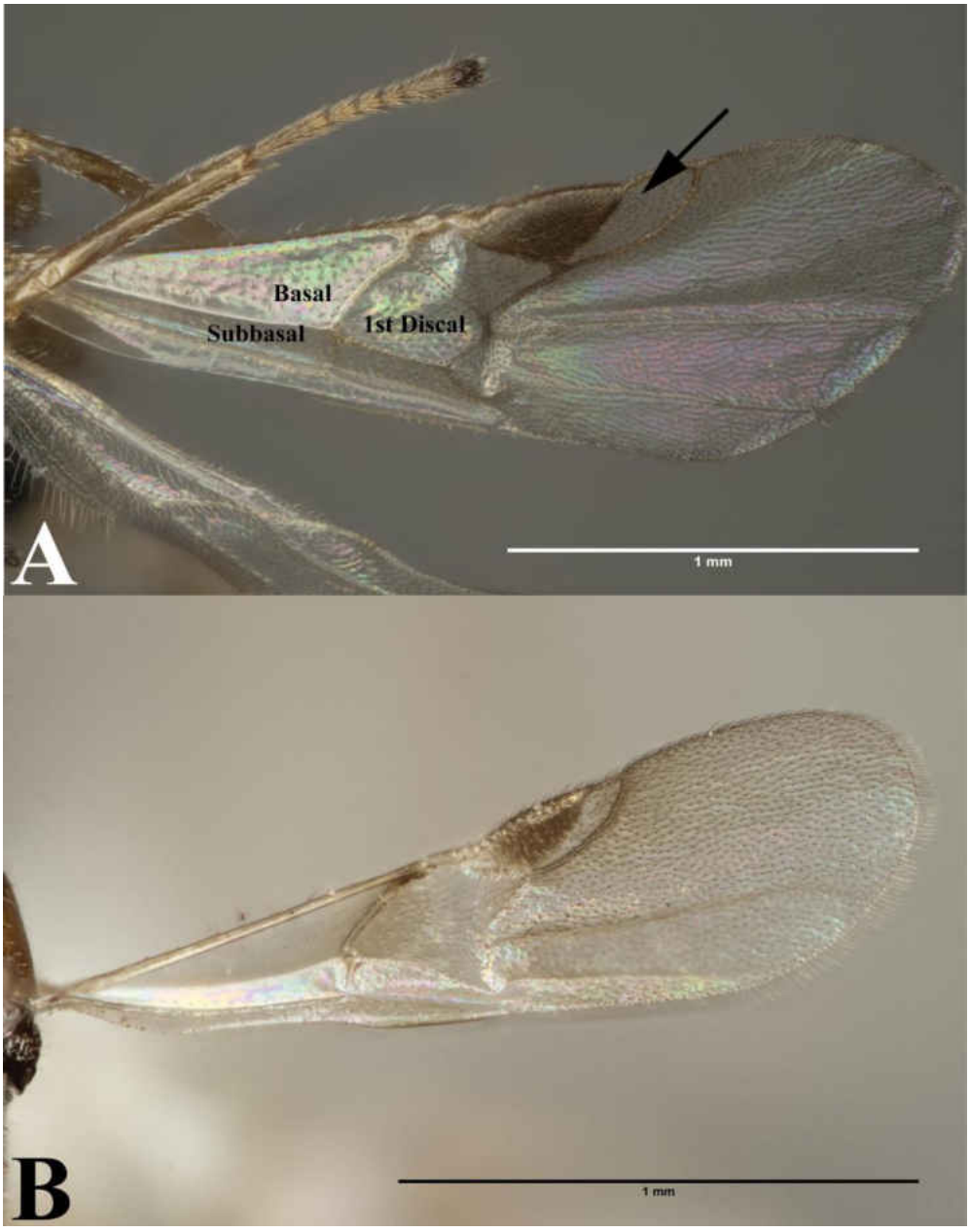

Figure 2.3. (A) Forewing of *Peristenus*; arrowing pointing to marginal cell; (B) Forewing of *Leiophron*.

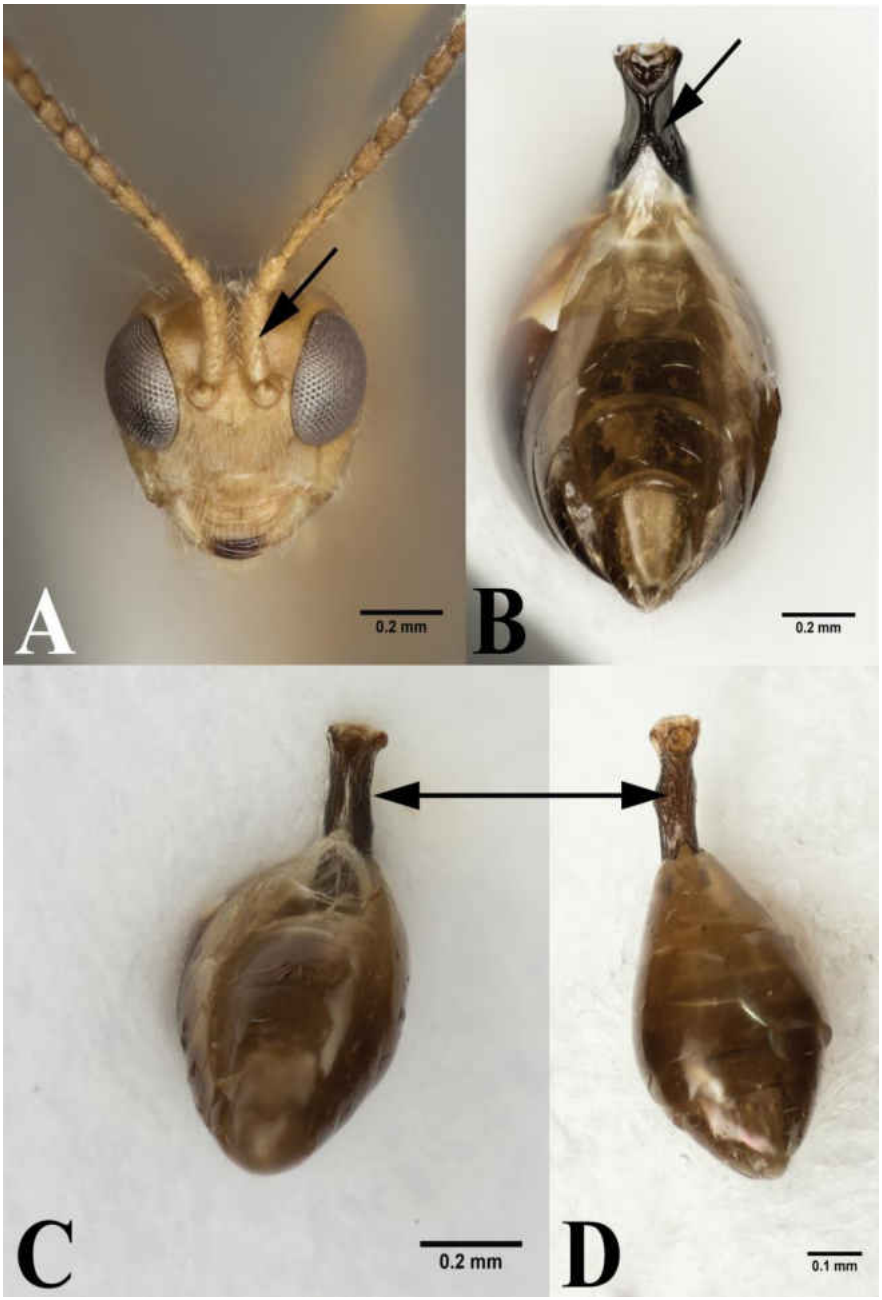

Figure 2.4. (A) Frontal view of *Leiophron reclinator* (*Mama mariae* **syn. n)**, arrow pointing to spiny scape of antennae; (B) Ventral view of *Peristenus* metasoma, arrow pointing to the partially fused petiole; Ventral view of *Leiophron* metasoma: (C) arrowing pointing to ventral petiole showing unfused sclerite at midline; and (D) arrowing pointing to completely fused sclerite at midline of petiole.

Tribe Euphorini Förster 1862

Diagnosis. Maxillary palp with five segments; labial palp with two to three segments; eye bare; first metasomal tergite petiolate; ovipositor slender and short, hardly protruding beyond metasoma; tarsal claws simple; forewing with marginal cell almost always equal or smaller than stigma (Fig. 2.3A); vein 3RSb (if present) strongly bent; vein r short or absent; vein 2M desclerotized; vein (RS+M)b absent; length of vein m-cu (if present) shorter than length of vein 2RS (Figs. 2.3A – B).

Genus *Peristenus* Förster 1862

***Peristenus* Foerster, 1862: 25; Shenefelt, 1969: 36 (as synonym of *Leiophron* Nees, 1818); Shaw, 1985: 332. Type species (by original designation): *Microctonus barbiger* Wesmael, 1835 [= *Leiophron pallipes* Curtis, 1833].**

Diagnosis. Antennal segments 16-33; labial palp with three segments; occipital carina complete or interrupted dorsally; notaulus well-defined, crenulate, posteriorly joining just before posterior margin of mesoscutum; forewing with marginal cell large, complete; basal, subbasal, and 1st discal cells of forewing similarly setose (Fig. 2.3A); veins (RS+M)a, 1m-cu, 2CUa, 2CUb of forewing fully developed (Fig. 2.3A); veins rs-m, 2-1A of forewing absent; vein M+CU of forewing unsclerotized; veins 1cu-a and 1-1A of hindwing fully present; first metasomal tergite widened apically, ventrally fused or touching basally (Fig. 2.4B); metasomal tergites behind first tergite smooth; second suture absent; second tergite with lateral fold; hypopygium medium-sized, densely setose; ovipositor sheath slender, short, and densely setose; ovipositor slender, distinctly curved downwards.

Biology. Koinobiont endoparasitoids of Miridae (Hemiptera). The early instar nymphs are parasitized and the mature parasite larva emerge from either the mature host nymphs or the adults.

Distribution. Cosmopolitan except for Antarctic, limited to Papua New Guinea in Australasia.

Genus *Leiophron* Nees, 1818

Leiophron **Nees, 1818: 303; Shenefelt, 1969: 35; Shaw, 1985: 326. Type species (designated by Viereck, 1914):** ***Leiophron apicalis*** **Haliday, 1833.**

Euphoriana **Gahan, 1913: 433; Shenefelt, 1969: 33; Shaw, 1985: 326. Type species (by original designation):** ***Euphonana uniformis*** **Gahan, 1913. Syn. by Loan, 1974.**

Euphoriella **Ashmead, 1900: 116; Shenefelt, 1969: 34; Shaw, 1985: 323. Type species (by monotypy & original designation):** ***Labeo incertus*** **Ashmead, 1887.**

Euphorus **Nees, 1834: 360; Shenefelt, 1969: 35; Shaw, 1985: 326*):* Type species (by monotypy):** ***Euphorus pallicornis*** **Nees, 1834.**

Mama **Belokobylskij, 2000: 256; Stigenberg et al. 2005: 590. Type species (by monotypy & original desgination):** ***Mama mariae*** **Belokobylskij, 2000. Syn.n.**

Diagnosis. Antennal segments 14-20; labial palp with two to three segments; occipital carina usually widely interrupted dorsally; notaulus usually absent; marginal cell of forewing small, incomplete, or absent; 1st discal cell of forewing often more setose than basal and subbasal cells (Fig. 2.3B); forewing vein 3SRb ending far beforewing apex; forewing vein (RS+M)a present or absent; forewing vein 2M present; forewing vein M+CU largely unsclerotized; forewing vein 1M usually thickened; forewing vein 1CUb sclerotized or unsclerotized; forewing veins 2CUa and 2CUb absent; hindwing vein cu-a partly present or absent*;* first metasomal tergite nearly parallel-sided or slightly widened apically, ventrally variable: largely open, separated by a split at the midline (Fig. 3C), largely touching, or entirely fused (Fig. 2.3D); second and third tergites without lateral fold and second metasomal suture absent; hypopygium small, straight ventrally and setose; ovipositor hardly visible, usually shorter than 0.25 times first tergite, slender and curved downwards.

Biology. Koinobiont endoparasitoids of nymphal Hemiptera (Miridae and Lygaeidae) and Psocodea (Psocidae). The early instar nymph of the host is parasitized and the mature larva emerges from the mature host nymph or adult.

Distribution. Cosmopolitan except for Antarctic, limited to Papua New Guinea in Australasia.

Conclusions

Using a multilocus phylogenetics approach and the most comprehensive taxon sampling of Euphorini to date, we were able to clarify the long standing taxonomic confusion within this tribe of economically important braconid wasps. The taxonomic uncertainty that has long impacted biological control studies of Euphorini is readily resolved with the revised generic

concepts presented here, which reflects the strongly supported phylogenetic analyses, therefore providing clear distinguishing characters for the two genera *Peristenus* and *Leiophron*. With a phylogenetic framework to build upon, the next step should focus on the world revision of tribe Euphorini, with a strong alpha taxonomic component, as many of the species used in this study were undescribed or have an unknown biology.

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CHAPTER THREE: INTEGRATIVE TAXONOMY IMPROVES UNDERSTANDING OF NATIVE BENEFICIAL FAUNA: REVISIONS OF THE NEARCTIC PERISTENUS PALLIPES COMPLEX (HYMENOPTERA: BRACONIDAE) AND IMPLICATIONS FOR RELEASE OF EXOTIC BIOCONTROL AGENTS

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Abstract

The Nearctic *Peristenus pallipes* complex (Hymenoptera: Braconidae) consists of two species-groups that are further divided into nine species, separated largely using ecological rather than morphological differences. The species are re-examined using an integrative approach using morphometric multivariate ratios, molecular (*COI* and *CytB*), and ecological data to test the validity of the nine species. The data supports only three valid species (*P. dayi* Goulet 2006, *P. mellipes* (Cresson 1872), and *P. howardi* Shaw 1999) rather than nine. New synonymies include: *P. braunae* Goulet 2006 under *P. dayi* Goulet 2006 **syn. n.**; *P. carcamoi* Goulet 2006, *P. otaniae* Goulet 2006, and *P. pseudopallipes* (Loan 1970) under *P. mellipes* (Cresson, 1872) **syn.n.**, and finally *P. broadbenti* Goulet 2006 and *P. gillespiei* Goulet 2006 under *P. howardi* Shaw 1999 **syn.n**. In light of these taxonomic revisions, the biology and distributions of the Nearctic *P. pallipes* complex are updated, resulting in three morphologically variable, widespread, multivoltine species rather than nine largely univoltine species with patchy distributions. The integrative taxonomic approach used here allowed for a more accurate delineation of native fauna and their potential to be competitively displaced by foreign biocontrol agents.

Introduction

Foreign biocontrol agents are often introduced intentionally to attack foreign invasive pests, or when no suitable native natural enemies can be used to suppress pest populations (van Driesche, 1994). However, extensive research on the biology (e.g. phenology, host-specificity) of foreign biocontrol agents is required prior to release, to ensure success of the control program. Poorly matched phenologies between the target pest and biocontrol agent can result in poor pest control (Boettner et al. 2000). Even worse, if host-specificity tests are not completed, biocontrol agents can cause unforeseen damage to nontarget species or native congeners occupying the same niche as a result of competitive displacement (DeBach & Sundby, 1963; Bennett, 1993; Schellhorn et al. 2002; van Driesche, 2008). Competitive displacement is even more of a concern when foreign agents are imported to control native pests, as their release will inevitably cause competition with the suite of natural enemies that have co-evolved with the pest (DeBach & Sundby, 1963; Bennett, 1993; Schellhorn et al. 2002). Displacement of native fauna may be a particularly important phenomenon in parasitoids that are specialized on the target pest (Xu et al. 2013) as they may have lost the ability to utilize alternate hosts. Therefore, understanding the possibility for interspecific competition among native and introduced parasitoid species is vital for reducing negative impacts associated with releasing foreign parasitoids for biocontrol. Displacement of native parasitoids due to foreign biocontrol agents is rarely reported in the literature, likely for two major reasons: (1) limited studies, particularly long-term studies post establishment of a biocontrol agent (Bennett, 1993), and (2) insufficient information on the native parasitoid community prior to release of a biocontrol agent, preventing comparative studies (Bennett, 1993). The latter is especially likely given that much of the parasitoid

community remains undescribed (Godfray, 1994) and taxonomists are not always consulted in biocontrol studies.

Parasitoids in the genus *Peristenus* Foerster (Braconidae: Euphorinae, Fig. 3.1) are found in most regions of the world except for Australia and the Neotropics. Currently there are 69 described species of *Peristenus* (Yu et al. 2012), all of which are endoparasitoids of plant bugs (Hemiptera: Miridae). The actual number of species is likely much higher, as only the European (Loan, 1974a), Oriental (Chen & van Achterberg, 1997; Shamim et al. 2008), Eastern Palearctic (Belokobylskij, 2000), and North American species (Loan, 1974b; Goulet and Mason, 2006) have been revised. The taxonomic status of *Peristenus* has fluctuated depending on the author, either recognized as a distinct genus (Shaw, 1987; Chen & van Achterberg, 1997; Stigenberg et al. 2015) or treated as a subgenus of *Leiophron* Nees (Tobias, 1986; Papp, 1992, Belokobylskij, 2000). The most recent phylogenetic study (Stigenberg et al. 2015) using morphological and molecular data supported *Peristenus* as a distinct clade and sister to *Leiophron*; however, taxon sampling was limited to five exemplars.

Figure 3.1. Freshly emerged adult *Peristenus mellipes*.

While most *Peristenus* have a partially complete occipital carina; members of the *Peristenus pallipes* species complex can be easily identified by the presence of a complete occipital carina, and are found in temperate to boreal Holarctic regions (Loan, 1974a; 1974b; Goulet and Mason, 2006). *Peristenus pallipes* Curtis was thought to be a single, common Holarctic species (Loan, 1974a; 1974b), but recent work has identified nine Nearctic and multiple yet to be described Palearctic species (van Achterberg and Goulet pers. comm.). *P. pallipes* is now treated as exclusively found in Europe whereas the North American specimens are treated as *Peristenus mellipes* (Cresson), the oldest name for North American endemics (Goulet & Mason, 2006).

The nine Nearctic species are further split into two species-groups: the *Peristenus dayi* group including two species (*P. braunae* Goulet 2006 and *P. dayi* Goulet 2006) and the

Peristenus mellipes group with seven species (*P. broadbenti* Goulet 2006, *P. carcamoi* Goulet 2006, *P. gillespie* Goulet 2006, *P. howardi* Shaw 1999, *P. mellipes* (Cresson, 1872), *P. otaniae* Goulet 2006, and *P. pseudopallipes* (Loan 1970)). The two species-groups were separated based on the density of punctures on the head, with the *dayi* group having large and dense puncturing, and the *mellipes* group with smaller and sparse punctures (Goulet & Mason, 2006). However, due to the lack of consistent morphological differences between species within the two species-groups, identification beyond the species-group level was largely based on generalized biogeographical distributions and peak flight times (Goulet & Mason, 2006). Table A3.1 lists the nine species along with their distributions, hosts, and life cycles based on Goulet and Mason (2006). The ecological information utilized to separate the species was largely generalized and thus calls into question the validity of the species. For example, *P. dayi* and *P. braunae* were considered not to have gene flow as populations were not found within 300 kilometers. However, extensive sampling across the intermediate area was not completed. Additionally, the peak flight times of these two species were considered diagnostic for species delimitation by Goulet & Mason (2006), with *P. dayi* in late May and *P. braunae* in late June to early July (Table A3.1). However, delayed host emergence in colder climates across the larger range of *P. braunae* would likely influence the average peak flight time, and thus may not be indicative of true phenological differences across species, but rather a result of climatic differences across the species' range.

Species of *Peristenus* attack early nymphal instars of mirids and kill their hosts in the late nymphal or adult stage (Loan, 1980). Thus, they have been used as biocontrol agents for major agricultural pests, such as the native Lygus bugs (*Lygus* Hahn) and the introduced *Adelphocoris lineolatus* (Goeze), that cause major economic damage into multiple North America crops, such as canola, and various pulses (Haye et al. 2005; 2006; Mason et al. 2011). Three European

species were introduced to North America to control native *Lygus* spp. and *A. lineolatus* populations in alfalfa and canola, amongst other crops (Day, 1996; Day et al. 1999; Mason et al. 2011). *P. digoneutis* Loan and *P. rubricollis* (Thomson) were introduced in eastern New Jersey and Delaware, respectively. Subsequent surveys have confirmed the establishment of both species in northeastern USA and eastern Canada (Broadbent et al. 1999; Day et al. 1990; Day et al. 1998; Day et al. 2008). *P. relictus* (Ruthe) (syn. *P. stygicus*), was released in California along with *P. digoneutis*, but only the former has confirmed establishment in Central California in recent surveys (Pickett et al. 2009; Pickett et al. 2013; Swezey et al. 2008). The main reason for the introductions of foreign biocontrol agents was that native *Peristenus* species were not considered abundant enough to control these mirid pests based on parasitism rate assessments in some localities (Day, 1987). There was an observed decline of native *Peristenus* species in eastern Québec, Canada after the introduction of *P. digoneutis*, which prompted the taxonomic and biological research of Goulet & Mason (2006). Recently, there has been interest to release foreign *Peristenus* species into Western Canada to control Lygus bugs (Fernández, 2016). However, competitive displacement of native parasitoids by foreign biocontrol agents remains a concern, prompting the current study to use integrative taxonomic approaches to re-examine the Nearctic *Peristenus pallipes* complex. Integrative taxonomy combines information from multiples sources, such as morphology, DNA, ecology, and behavior (Dayrat, 2005; Schlick-Steiner et al. 2010). This provides a more holistic taxonomic approach using multiple independent lines of evidence, and has largely improved cryptic species delimitation (Boring et al. 2011; Ceccarelli et al. 2012; Gebiola et al. 2012; Baur et al. 2014; Grossi et al. 2014; Namin et al. 2014; Schwarzfeld & Sperling, 2014; Zhang et al. 2014).

Thus, the main objective of this study was to test the species and species-group hypotheses put forth by Goulet & Mason (2006) for the Nearctic *Peristenus pallipes* complex using a combination of morphometrics, molecular, and ecological data. We test the validity of the nine species based on a phylogenetic species concept (monophyly) (Baum, 1992) in combination with a distinct barcoding gap (greater interspecific genetic distances than intraspecific) (Meyer & Paulay, 2005). Additionally, spatial and temporal specimen data are used in combination with phylogenetic patterns to examine possible intra-clade structuring associated with phenological information. Finally, we utilize a multivariate analysis of quantitative morphological characters to provide an additional independent test of species validity. We revise the two species-groups, synonymize species that are not supported, provide a key to the three native North American species of *Peristenus*, and update the biology and distribution records for the three valid species. Additionally, we discuss the implications of this study for importation of foreign parasitoids, with a focus on Western Canada. We also make recommendations for the inclusion of integrative taxonomic research prior to the release of biocontrol agents, which has global implications for classical biocontrol programs. Accurate identification of the Nearctic *Peristenus pallipes* complex will facilitate studies on their population dynamics with hosts and crops, potentially prevent extirpation and extinction of native beneficial insects, and contribute to a better understanding of the interactions of native species with foreign agents in classical biocontrol.

Materials and Methods

Sample Collection

Specimens of adult species of *Peristenus* were borrowed from the following institutions and curators: the Canadian National Collections of Insects (CNCI, J. Fernández-Triana), and

University of Guelph Insect Collections (DEBU, S. Paiero). Paratypes were borrowed when available and DNA extracted for inclusion in the analyses (Table A3.2). Additional specimens were collected as adults using sweep nets, or reared out from parasitized nymphs sampled in Manitoba, Ontario, and Alberta during May to August of 2013-2015 in various agricultural fields where the hosts can be found and preserved in 95% EtOH. Species were initially identified using a combination of morphological and ecological characters outlined in Goulet & Mason (2006). Outgroups included *Euphoriella* sp. and *Leiophron* spp, the latter which is sister to *Peristenus* (Stigenberg et al. 2015), and two specimens of *Peristenus relictus*, an European species that is not in the *P. pallipes* complex. A list of the specimens utilized in this study is provided in Table A3.2. For ease of interpretation of results, specimen information was added to taxon labels for the phylogenetic analyses, including province or state locality, date of collection, and initial identification based on the characters outlined in Goulet & Mason (2006). Additional exemplar specimens with sequences for the barcoding region of *COI* were obtained from BOLD Systems (http://www.boldsystems.org/) to increase taxonomic sampling across a larger biogeogrpahic range.

Morphometrics Analysis

A subset of sequenced female specimens along with identified specimens that failed to generate molecular data were selected for morphometrical analysis. Only females were used as most type specimens are female, females are more abundant than males, and to prevent any analytical issues that may be caused by sexual dimorphism. The chosen 40 female specimens were photographed using a Canon 7D Mark II with a Mitutoyo M Plan Apo 10x objective mounted onto the Canon EF Telephoto 70 – 200mm zoom lens, and the Canon MT–24EX Macro

Twin Lite Flash with custom made diffusers to minimize hot spots. Measurements were taken using the average of three measurements with ImageJ150 (Schneider et al. 2012) and/or a Nikon SNZ18 stereomicroscope with an ocular micrometer. A detailed list of measurements is presented in Table 3.1 and shown in Fig. 3.2. The multivariate ratio analysis (Baur & Leuenberger, 2011) was applied in R (R Core Team, 2016) with modified scripts (this study) as outlined in Baur et al. (2014). The script files can be accessed at the Dryad Digital Repository (http://datadryad.org/, doi:10.5061/dryad.vv183).

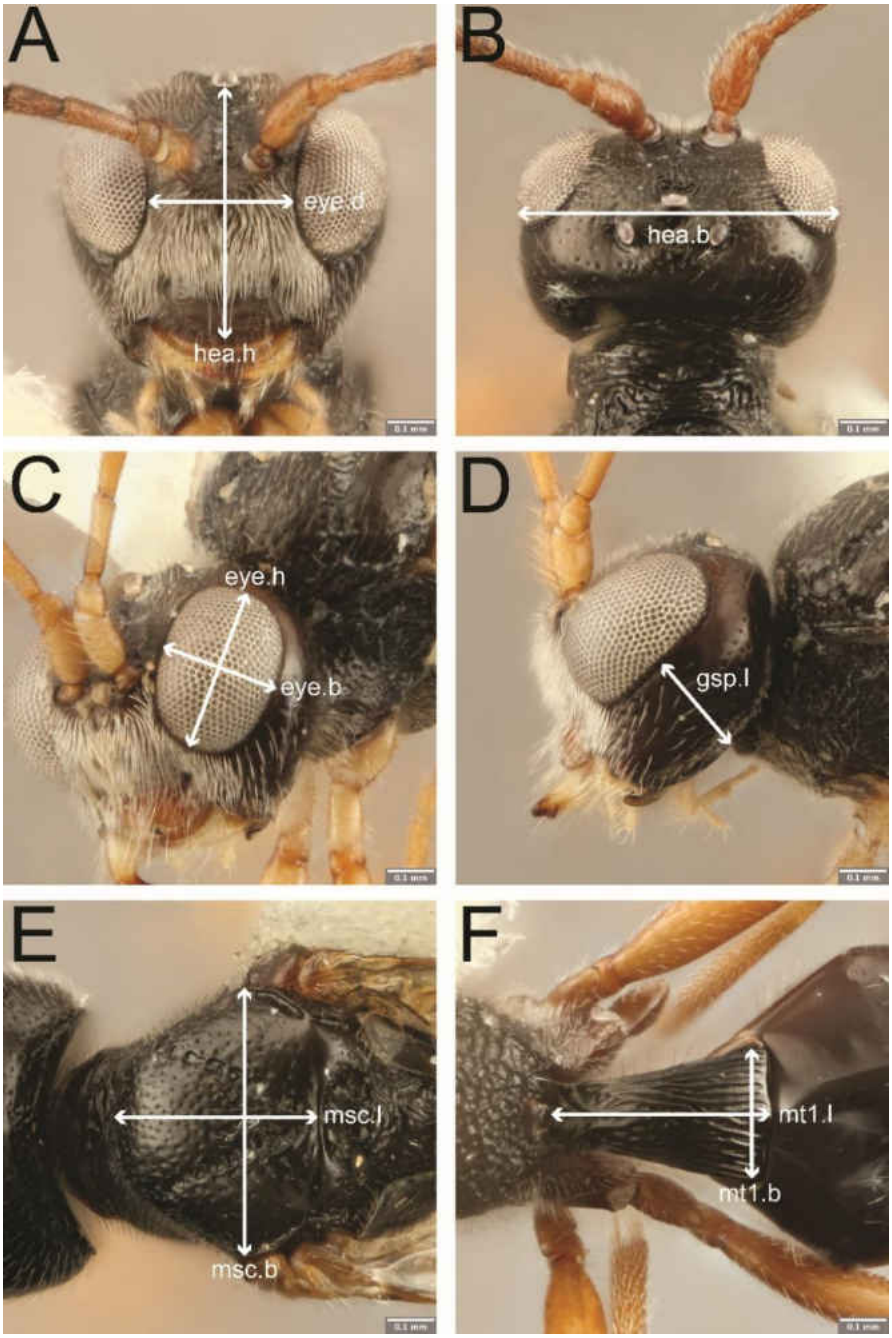

Figure 3.2. (A, B, F) *Peristenus dayi* ♀; (C,D, E) *Peristenus mellipes* ♀. (A) Frontal view of the head; (B) dorsal view of the head; (C) anteriolateral view of the eye; (D) dorsal view of the mesoscutum; (E) lateral view of the head; (F) dorsal view of metasomal tergite 1. Morphometrics variables: minimum eye distance (eye.d); head height (hea.h); head breadth (hea.b); eye height (eye.h); eye breadth (eye.b); mesoscutum length (msc.l); mesoscutum breadth (msc.b); genal space length (gsp.l); metasomal tergite 1 length (mt1.l); maximum metasomal tergite 1 breadth (mt1.b).

Table 3.1. Abbreviations and definitions of the 10 morphological characters used for the morphometrics analysis of *Peristenus pallipes* complex.

Abbreviation	Character name	Definition	Magnification
eye.b	Eye Breadth	Greatest breadth of eye, viewed at an angle in which both anterior and posterior margins are in focus (Fig. 3.1C)	100x
eye.d	Eye distance	Shortest distance between eyes, frontal view (Fig. 3.1A)	100x
eye.h	Eye height	Greatest length of eye height, viewed at an angle in which both dorsal and ventral margins are in focus (Fig. 3.1C)	100x
gsp.l	Genal Space	Length of the genal space taken midway between the dorsal and ventral margins of the eye from the posterior edge at a 90° to the occipital carinae, lateral view (Fig. 3.1D)	100x
hea.b	Head breadth	Greatest breadth of head, dorsal view (Fig. 3.1B)	100x
hea.h	Head height	Distance between lower edge of clypeus and lower edge of anterior ocellus, frontal view (Fig. 3.1A)	100x
msc.b	Mesoscutum breadth	Greatest breadth of mesoscutum just in front of level of tegula, dorsal view (Fig. 3.1E)	100x
msc.l	Mesoscutum length	Length of mesoscutum along median line from posterior edge of pronotum to posterior edge of mesoscutum, dorsal view (Fig. 3.1E)	100x
mt1.b	Metasomal tergite 1 breadth	Greatest breadth of metasomal tergite 1 at the posterior margin, dorsal view (Fig. 3.1F)	100x
mt1.l	Metasomal tergite 1 length	Medial length from the base of metasomal tergite 1 to the posterior margin, dorsal view (Fig. 3.1F)	100x

DNA Protocols

Genomic DNA was extracted from mounted or EtOH preserved specimens following the DNeasy™ Tissue Kit protocol (Qiagen, Valencia, CA, U.S.A.). Petioles were separated from mesosomas to ensure buffer penetration during tissue lysis, and the two body parts were mounted onto the same point post-extraction. Voucher specimens were deposited in CNCI. Two genes were amplified: Mitochondrial Cytochrome Oxidase I (*COI*) using universal primers LCO1490 (5'- GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994); and Cytochrome B (*CytB*) using CytB F (5' -TCT TTT TGA GGA GCW ACW GTW ATT AC-3') and CytB R (5 '- AAT TGA ACG TAA AAT WGT RTA AGC AA -3') (Belshaw & Quicke, 1997). The faster rate of evolution of the mitochondrial genes compared to nuclear DNA makes mtDNA ideal for separating closely related species, and both genes are frequently used for species delimitation of Braconidae, including Euphorinae (Stigenberg and Ronquist, 2011; Ceccarelli et al. 2012). All PCRs were performed on a Bio-Rad MyCyclerTM thermal cycler, using approximately 1µg DNA extract, 1X Standard Taq Buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, pH 8.3, New England Biolabs, Ipswich, Massachusetts, U.S.A.), 200 µM dNTP (Invitrogen, Carlsbad, California, U.S.A.), 4 mM $MgSO_4$, 400 nM of each primer, 1 unit of Taq DNA polymerase (New England Biolabs), and purified water to a final volume of 25 µl. Amplicons of COI were generated with an initial denaturation of 1 min at 95ºC, followed by 35 cycles of 95ºC for 15 s, 49ºC for 15 s and 72ºC for 45 s, and a final elongation period of 4 min at 72ºC. Amplicons of *CytB* were generated with an initial denaturation of 2 min at 95ºC, followed by 35 cycles of 95ºC for 15 s, 45ºC for 15 s and 72ºC for 30 s, and a final elongation period of 4 min at 72ºC. Reaction

products were cleaned with Agencourt CleanSEQ magnetic beads and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) and the Applied Biosystems 3730xl DNA Analyzer at the University of Kentucky, Advanced Genetic Technologies Center (UK-AGTC). Contigs were assembled and edited using Geneious version 8.18 (Kearse et al. 2012), and alignment was conducted using MUSCLE (Edgar, 2004) and then hand corrected using reading frames as a guide in BioEdit (Hall, 1999). Sequences obtained from this study were deposited in GenBank (See Table A3.2).

Phylogenetic Analyses

The two genes were concatenated for the Bayesian analysis, which was performed using Mr. Bayes version 3.6.11 (Ronquist et al. 2012) on CIPRES Science Gateway (Miller et al. 2010). Two independent searches and four chains for 20,000,000 generations and sampling every 1000, with 10% burnin discarded. The dataset was not partitioned based on nucleotide position and as it would limit the amount of data needed for accurate parameter estimation. The best fitting model of molecular evolution was tested using jModelTest2 (Darriba et al. 2012), and the general time-reversible model, with a parameter for invariant sites and rate heterogeneity modelled under a gamma distribution (GTR+I+G) was chosen based on the Bayesian Information Criterion (BIC). The concatenated dataset can be accessed at the Dryad Digital Repository (http://datadryad.org/, Accession # doi:10.5061/dryad.vv183). Intra- and interspecific genetic distances were calculated using MEGA version 7.0 (Kumar et al. 2016) using the Kimura-2-parameter model (Kimura, 1980). The phylogenetic trees were visualized in FigTree v1.4.2 (Rambaut, 2012) and enhanced using InkScape 0.91 (The Inkscape Team, 2016).

Results

Phylogenetic Analyses

The concatenated analysis was performed on 123 exemplars, with 122 taxa amplified for *COI* (579bp) and 31 for *CytB* (397bp). There was some difficulty amplifying *CytB* sequences, particularly for pinned type material. Of the COI sequences, 81 were downloaded from BOLD Systems (http://www.boldsystems.org/). The Nearctic *P. pallipes* complex was recovered as a monophyletic clade with strong support. The two species-groups (*P. dayi* and *P. mellipes*) recognized by Goulet & Mason (2006) were also recovered as monophyletic (Fig. 3.3), however, only three of the nine delineated species were supported.

Within the *P. dayi* species-group, *P. dayi* and *P. braunae* were recovered as paraphyletic with respect to each other, indicating only one valid species (Fig. 3.3). Genetic distances also supported only one species within the dayi species-group, as the average intraspecific distance was 1.7% in *COI* and 0.8% in *CytB* (Fig. 3, Table A3.3A), whereas interspecific distances between other clades within the *P. pallipes* complex ranged from 9.7 – 10.2 (Table A3.3B). There were no clear phylogenetic patterns based on spatial or temporal data, such that specimens from across all localities and early and late flight times were recovered in paraphyly. Thus, only one species is supported for the *P. dayi* species-group, thereby invalidating the species-group.

Within the *P. mellipes* species-group, two distinct clades were recovered, labeled A and B (Fig. 3.3). Clade A included all specimens identified as *P. mellipes, P. pseudopallipes*, *P. otaniae*, and *P. carcamoi*; however, they were all recovered as paraphyletic with respect to each other, indicating only one valid species among the four. The average intraspecific distances for the *mellipes* clade were 1.6% for *COI*, and 0.3% for *CytB* (Fig. 3.3, Table A3.3A), whereas

interspecific distances between other clades within the *P. pallipes* complex ranged from 4.2 – 10.2 (Table A3.3B), indicating a distinct barcoding gap. Similar to *P. dayi*, there was no phylogenetic spatial or temporal patterns, indicating one widely distributed species. This clade is named as *P. mellipes*, which is the oldest synonym for the members of the Nearctic *P. pallipes* complex included in this clade. In the second clade (Fig. 3.3, Clade B), *P. broadbenti*, *P. gillespiei,* and *P. howardi* were recovered together and paraphyletic with respect to each other with average intraspecific distances of 0.9% in *COI* and 0.2% in *CytB*. (Fig. 3.3, Table A3.3A), indicating only one valid species among the three. The smallest distance to other recovered clades was 4.2% in *COI* to *P. mellipes* (Table A3.3B), again signifying a distinct barcoding gap. The included exemplars ranged from Nevada to Alberta and Idaho, indicating a widely distributed species found from mid-June to early August. The clade is named *P. howardi,* as it is the senior synonym of the three included species epithets.

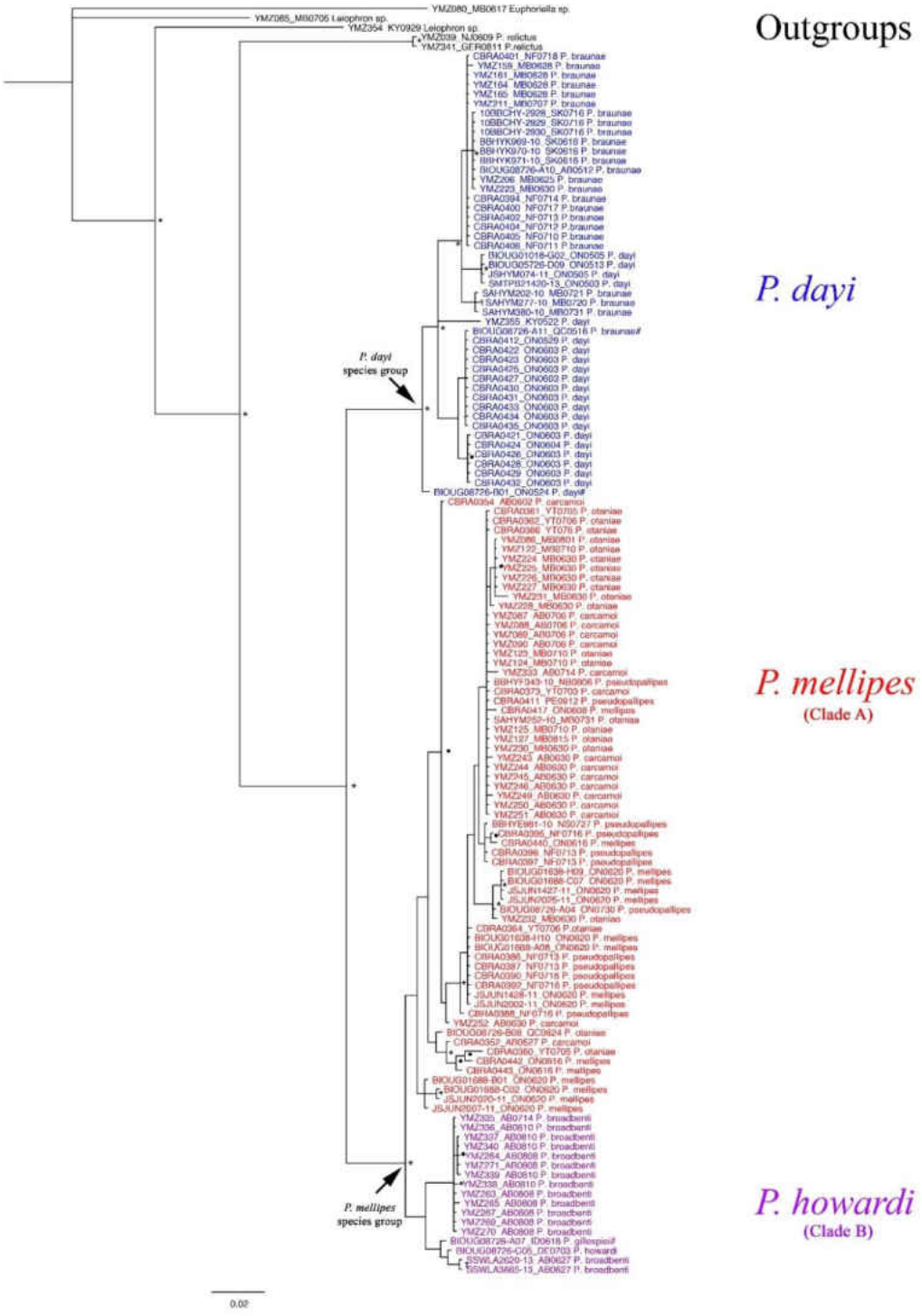

Figure 3.3. Inferred concatenated topology from the Bayesian analysis of COI and CytB. Posterior probabilities ≥0.95 are indicated by an asterisk; posterior probabilities between 0.90 and 0.94 are indicated by a black dot. Arrows indicate species groups, and clades A and B represent *Peristenus mellipes* and *Peristenus howardi*, respectively.

Morphometric Analyses

The three species supported by the molecular data (*P. dayi*, *P. mellipes*, *P. howardi*) were examined using a multivariate ratio analysis. Assignment to species was purposely avoided and groups were assigned based on molecular operational taxonomic units (MOTUs) according to the results of the molecular analysis. A series of shape PCAs (Principle Component Analysis) were performed to determine how well the MOTUs were supported by variation in shape. A PCA is appropriate for this study because it does not require *a priori* assignment of specimens, but instead assumes all MOTUs belong to a single group, thus avoiding bias in respect to particular groupings (Laszlo et al. 2013). Only the first and second shape PC were informative and accounted for 56.8% of the variation (Fig. 3.4A). The two species-groups (*P. dayi* and *P. mellipes*) were separated based on the 1st principal component, however the two MOTUs within the *P. mellipes* group (*P. mellipes* and *P. howardi*) were not (Fig. 3.4A). The second principal component showed no separation between the two species-groups. The first principal component was plotted against isometric size (Fig. 3.4B), which is defined as the geometric mean of all body measurements (see Baur & Leuenberger, 2011). There was no correlation between shape and size, indicating little to no allometry (Baur & Leuenberger, 2011), which indicates that differences in measured ratios across species are independent of body size.

PCA and allometry ratio spectrums are generated to show the best characters for discriminating putative species. Characters on opposite ends of the PCA spectrum show the most variation and therefore the best likelihood of diagnosing species, whereas characters closer together contribute very little to variation and should not be used. The allometry ratio spectrum is used in a similar manner, however the further characters are from each other the greater the

allometry. The ratio spectrum of the first principal component showed that most of the variation was explained by ratios such as *eye.d:eye.b* or *eye.d:eye.h* (Fig. 3.4C). The allometric ratio spectrum showed that the ratios *mt1.b:eye.h* and *mt1.b:eye.b* contributed the most to allometry within the groups (Fig. 3.4D). The variables that correspond to the separation of the two species-groups (Fig. 3.4C) were different from the variables that showed the greatest allometry (Fig. 3.4D), indicating that these characters are different due to shape and not size (Laszlo et al. 2013). A LDA (Linear discriminant analysis) ratio extractor was then used to determine which ratios would be the best at separating the two groups: The most discriminating ratio was *eye.d:eye.b*, with the second best being *eye.h:gsp.l* (see ranges for the LDA ratios in Table A3.4), which are used in the identification key to help facilitate species identification (see below).

Thus, the morphometrics data supports only two species (*P. dayi* and *P. mellipes*), corresponding to the original species-groups put forth by Goulet & Mason (2006). This result contrasts with the three species supported by the molecular and ecological and biogeographical data. Thus, the two species within the *P. mellipes* species-group are truly cryptic, as the molecular data provides abundant evidence to support separation of the species based on a distinct barcoding gap and the phylogenetic species concept. The distributions and flight times of the three supported *Peristenus* species are expanded to reflect the current taxonomic revisions (Table 3.2). The ecological data further support separation between *P. mellipes* and *P. howardi* as the range of the latter species is restricted to western North America, ranging from western Canada down to California. Additionally, *P. howardi* has been reared from *Lygus hesperus*, which is only a western species (Goulet & Mason, 2006).

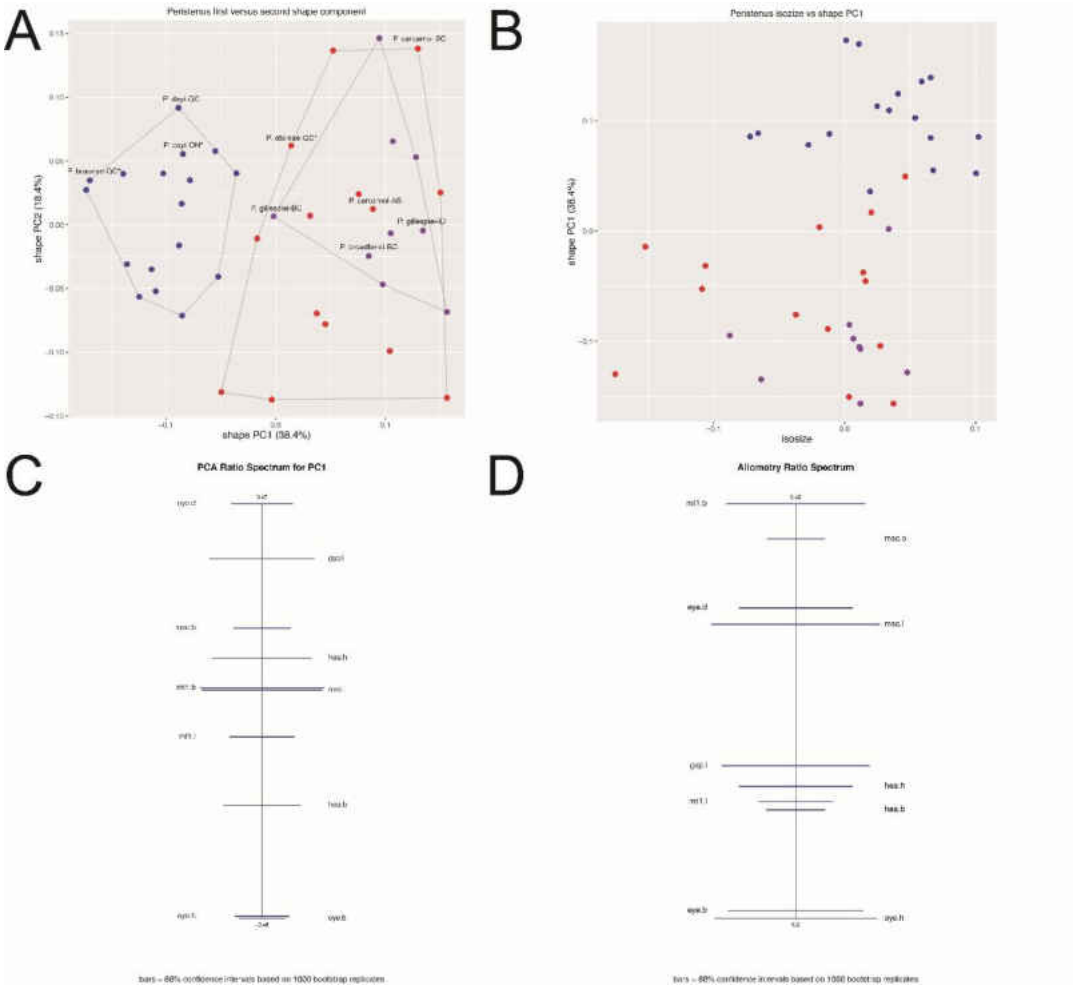

Figure 3.4. Size and shape analysis of ♀ *Peristenus* using all variables. (A, B) Blue, *Peristenus dayi*; red, *Peristenus mellipes*; purple, *Peristenus howardi*: (A, B) Shape principal component analyses (PCA): (A) scatterplot of first against second shape principal component (PC); (B) scatterplot of isosize against first shape PC. The variance explained by each shape PC is shown in parentheses. (C, D) Ratio spectra: (C) PCA ratio spectrum; (D) allometry ratio spectrum. Horizontal bars in the ratio spectra represent 68% bootstrap confidence intervals based on 1000 replicates.

Key to the Nearctic Peristenus pallipes Complex

Notes to the key: The morphometric ratios apply to >95% of the specimens, with only minor overlap. The coloration for *P. mellipes* and *P. howardi* are consistent, and locality can be further used to separate the species.

1. Punctures large and dense between inner eye margin and lateral ocellus (Fig. 3.5A). Metatibia testaceous or pale reddish brown, and metatarsomere 1 testaceous (Fig. 5E). Female minimum eye distance approximately 1.25x the breadth of the eyes (mean eye.d:eye.b = 1.24; range =1.14–1.38). Found across Canada and northern USA from California to Nova Scotia (Table 3.2)

……..……..……..……..***Peristenus dayi*** Goulet

1'. Punctures fine and scattered between inner eye margin and lateral ocellus (Fig. 3.5B). Metatibia dark brown to black in apical half of the dorsal surface, metatarsomere 1 darker than following tarsomeres (Fig. 3.5F). Female minimum eye distance approximately the same as the breadth of the eyes (eye.d:eye.b = 1.05; range =0.90–1.24).

……...……..……..……**2**

2. Clypeus (Fig. 3.5C), metasoma, and metacoxa light brown (Fig. 3.5F), found mainly east of the Rocky Mountains, extending from the Yukon Territories to Newfoundland (Table 3.2)

……..…….....***Peristenus mellipes*** (Cresson)

2’. Clypeus (Fig. 3.4D), metasoma, and metacoxa black (Fig. 3.5G), found mainly west of the Rocky Mountains, extending from southern Alberta to pacific coast down to Nevada (Table 3.2)

……..…….....***Peristenus howardi*** Shaw

Table 3.2. Updated list of Nearctic species of the *Peristenus pallipes* complex, their distribution, host, and peak flight time as a result of this study. New provincial/state records are designated by an asterisk (*).

Species	Species group	Distribution	Flight Period	Voltinism	Host(s)	Provincial/State Record
P. dayi	invalid	Widespread across North America	May - Aug	Bivoltine	*Adelphocoris lineolatus*, *Lygus lineolaris*	AB, AK, BC, CA, CO, DE, MB, NB, NF*, NJ, NS, NT, NY, ON, QC, SK, UT
P. mellipes	*mellipes*	Widespread across North America	May - Sep	Bivoltine	*A. lineolatus*, *L. lineolaris*, *Lygus* spp.	AB, BC, CO, CT, DE, GA, IL, KS, MA, MB, ME, MI, MO, MS, NB, NC, NF, NS, NJ, NY, OH, ON, QC, SK, VA, YT*
P. howardi	*mellipes*	Western North America	May - Sep	Multivoltine	*L. hesperus*, *Lygus* spp.	AB, BC, CA, ID, MT, NV, OR, WA, WY

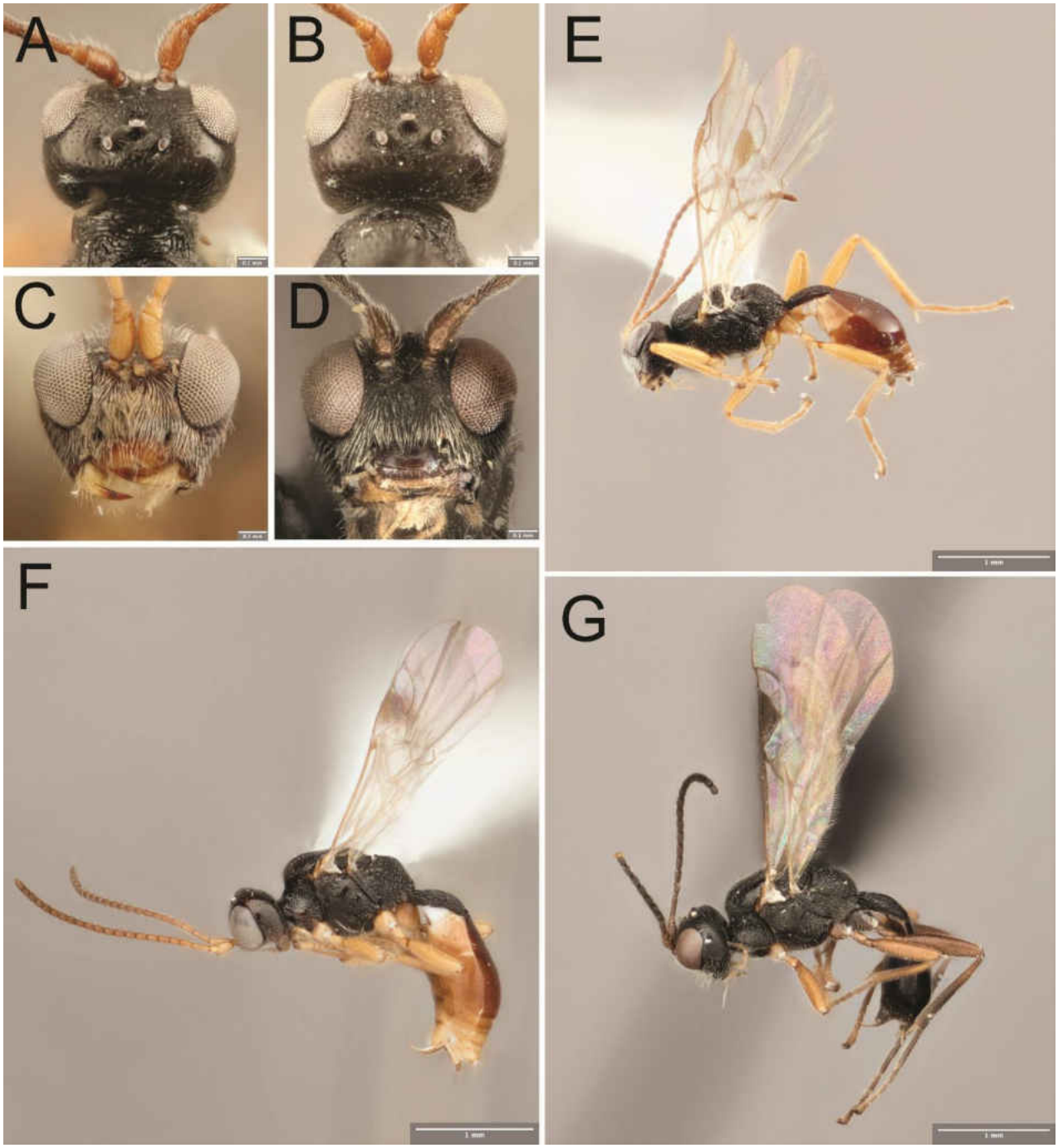

Fig. 3.5. (A, B) Dorsal view of the ♀ head: (A) *Peristenus dayi*, (B) *Peristenus mellipes*. (C, D) Frontal view of the ♀ head: (C) *Peristenus mellipes*, (D) *Peristenus howardi*. (D–F) Lateral habitus of ♀: (E) *Peristenus dayi*, (F) *Peristenus mellipes*, (G) *Peristenus howardi*.

Discussion

Members of the *Peristenus pallipes* complex are difficult to distinguish due to high intraspecific and low interspecific morphological variation (Goulet & Mason, 2006). Using the integrative taxonomy approach of combining morphological characters, molecular evidence, and ecological data, we have re-examined the Nearctic *P. pallipes* complex to refine the species-groups, and to test the validity of the species within each group. Our results did not support the species concepts put forth by Goulet & Mason (2006), in which each of the species would have resulted in a monophyletic clade with specific geographical and/or peak flight time patterns as seen in Table A3.1. The morphometric, molecular, and ecological data support the synonymy of *P. braunae* as a junior synonym of *P. dayi* **syn. n.**. The seven members within the *P. mellipes* group (*P. broadbenti*, *P. carcamoi*, *P. gillespiei*, *P. howardi, P. mellipes, P. otaniae*, *P. pseudopallipes*) are split into two species, with *P. carcamoi*, *P. otaniae*, and *P. pseudopallipes* synonymized under *P. mellipes* **syn. n.**; and *P. broadbenti* and *P. gillespiei* synonymized under *P. howardi* **syn. n.**. Interestingly, in regions like Lethbridge, Alberta where multiple species occur, there seems to be evidence for niche partitioning: *P. mellipes* emerge from early May to the end of June to attack the first generation *Lygus*, whereas *P. howardi* emerge later from late June to early September and attack the second generation (Fernández, 2016). Future studies will focus on the two *Peristenus* species on a population level, rather than at the species level, to examine microevolutionary forces at a finer scale that may contribute to reproductive isolation and maintenance of these two species (See Chapter 4).

The diversity of *Peristenus*, in particular the *Peristenus pallipes* complex is undoubtedly much higher than currently known and the entire group is in need of taxonomic revision. While

the Palearctic fauna is beyond the scope of this paper, the relationships between the Holarctic *Peristenus* fauna is integral to resolving the *Peristenus pallipes* complex as a whole (van Achterberg & Goulet, unpublished data). A similar approach using integrative taxonomy is highly recommended as molecular evidence is vital as a screening process to avoid over-splitting based on inconsistent morphological data. Multivariate morphometrics based on characters on mainly the head was used for this study, but perhaps geometrics morphometrics of the wings or interference patterns might improve species delimitation when combined with molecular and ecological data (Villemant et al. 2007; Shevtsova & Hansson, 2011).

This taxonomic revision has interesting implications for the impact that European *Peristenus* have had on native *Peristenus*. The distribution of the three *Peristenus* species were expanded as a result of this study, demonstrating a very wide geographical range, especially for *P. mellipes* and *P. dayi*. While these species may have been locally extirpated from parts of eastern Canada (Goulet & Mason 2006), they remain more abundant in western Canada. Local extirpation is thus less of an issue, as re-introductions from other regions could be possible, making the two species less susceptible to extinction. However, whether or not local extirpation of native *Peristenus* has affected more regions is unknown, as only limited sampling has been done across North America (Goulet & Mason, 2006). Cases of competitive displacements as results of biocontrol agent introductions have been reported in other groups of braconids, such as the displacement the native *Cotesia glomerata* L. by the introduced *Cotesia rubecula* L. in northeastern North America (van Driesche, 2008); or the European *Aphidius ervi* Haliday outcompeting native *Praon pequodorum* Viereck in the span of 20 years (Schellhorn et al. 2002). While the decline of native *Peristenus* as a result of competitive displacement has largely been

anecdotal (Goulet & Mason, 2006), it still serves as a warning to future biocontrol studies on the inadvertent effects of foreign parasitoids on native beneficial insect fauna.

The integrative taxonomic approach used here allowed for a more accurate circumscription of native fauna and their potential to be competitively displaced by foreign biocontrol agents. Thus, the renewed interest in releasing the European *P. digoneutis* to western Canada should be considered carefully, as the release may have detrimental effects on native *Peristenus* populations. In particular, *P. howardi* may be more susceptible to extirpation and possibly extinction as it has a more limited distribution. Using this study as an example, researchers in biocontrol should continue to work closely with taxonomists both pre- and post- release of foreign agents, as well as augmentation and conservation of native natural enemies. However, this is particularly important when exotic biocontrol agents are imported to control native pests. Native pests have their suite of natural enemies, and thus foreign agents create new competitive forces for these natural enemies. This is in direct contrast to more classical biocontrol programs where the pest is exotic and thus benefits from enemy-free space in the new environment (Holt and Lawson, 1993). Finally, post-release monitoring of pest and native population dynamics is highly recommended to better understand the impacts of foreign agents on local fauna.

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CHAPTER FOUR: HOST-PARASITOID INTERACTIONS AND ECOLOGICAL SPECIATION WITHIN THE *PERISTENUS PALLIPES* COMPLEX (HYMENOPTERA: BRACONIDAE) USING GENOMIC DATA

Abstract

Ecological speciation is often observed in phytophagous insects and their parasitoids due to divergent selection caused by host associated or temporal differences. However, most previous studies have utilized limited genetic markers or distantly related parasitoids to look for drivers of speciation. In our study we focus on closely related species of *Lygus* bugs and two sister species of *Peristenus* parasitoid wasps. Using mitochondrial DNA *COI* and genome wide SNPs generated using ddRADSeq, we tested for potential effects of host-associated differentiation (HAD) or allochrony in this system. While three species of *Lygus* are clearly identified with both *COI* and SNPs, no evidence of HAD or allochrony was detected. Two *Peristenus* sister species were identified by both sets of markers and exhibited temporal separation, as *P. mellipes* emerges early in June and attacks the first generation of *Lygus*, while *P. howardi* emerges later in August and attacks the second generation of their hosts. This is one of the few studies to examine closely related hosts and parasitoids to examine drivers of diversification. Given the results of this study, the *Lygus-Peristenus* system demonstrates allochrony as a driving force for ecological speciation, which could indicate higher parasitoid diversity in regions of multivoltinism of hosts if allochrony is common. This study also demonstrates the importance of systematics to studies of parasitoid speciation, particularly careful delimitation of cryptic species, host rearing to obtain accurate records, and genomic scale data for examining any population level differences among closely related taxa.

Introduction

A growing number of evolutionary studies have focused on ecological speciation in sympatry, in which new species arise as a result of ecologically-driven divergent selection (Egan et al. 2015; Hood et al. 2015; Nosil et al. 2002; Rundle & Nosil 2005; Schluter 2009). Ecological speciation is often observed in herbivorous insects in the form of host associated differentiation (HAD), where specialists diverge through phenological or host shifts to avoid competition and/or predation, leading to the separation and eventual formation of new species (Dres & Mallet 2002; Forbes et al. 2017). Another well-documented factor of ecological speciation is divergence in the breeding time, or allochrony, over timescales ranging from days, seasons, or even years (Taylor & Friesen 2017). Allochrony can contribute to divergence alone or concurrently with traits such as host preference to reinforce divergence along the speciation continuum (Egan et al. 2015; Feder et al. 1994; Taylor & Friesen 2017). Although allopatric populations are often defined by spatial differentiation, populations with overlapping distributions and phenological differences can also be argued as allopatric in a temporal scale (Taylor & Friesen 2017). Most documented cases of allochronic speciation among phytophagous insects involve seasonal separation of breeding time after a host shift to better synchronize with host phenology, contributing to reproductive isolation (Egan et al. 2015; Feder et al. 1994; Nosil et al. 2002; Stireman et al. 2005). These phenological shifts are often associated with genes controlling diapause duration, timing of diapause termination and circadian rhythms, which could contribute to divergent selection forces that ultimately drives ecological speciation (Ragland et al. 2017; Ragland et al. 2011; Ragland et al. 2012; Taylor & Friesen 2017).

Numerous studies have shown that described insect herbivore species are often multiple genetically divergent cryptic lineages, each specializing on a subset of the full host-plant range

(Dres & Mallet 2002; Peccoud et al. 2009; Powell et al. 2014). As such, many species previously thought to be generalists are actually cryptic specialists. This is an important distinction as true generalist species feed on a variety of host plants indiscriminately, while cryptic specialists exhibit host preferences but were overlooked due to morphological similarities. HAD has been recorded from speciose insect families across multiple orders (Antwi et al. 2015; Leppanen et al. 2014; Sword et al. 2005), further suggesting HAD as an important driver of speciation that resulted in the biodiversity that we see today. In addition, HAD can have rippling effects at higher trophic levels, resulting in divergence of parasitoids in the form of cascading/sequential HAD (Forbes et al. 2009; Hood et al. 2015; Nicholls et al. 2018; Stireman et al. 2006). As many parasitoids are also cryptic specialists that are tightly linked to the phenology of their hosts, cascading HAD on speciose lineages of herbivores could have also resulted in the radiation of these hyperdiverse lineages of parasitoids (Forbes et al. 2009; Hood et al. 2015; Stireman et al. 2006). However, many of the past studies on HAD and sequential HAD were limited by the number of molecular markers (Antwi et al. 2015; Hood et al. 2015; Leppanen et al. 2014; Nicholls et al. 2018; Stireman et al. 2006), thus providing insufficient molecular characters to accurately track species-level differentiation. In addition, few studies have focused on parasitoids as the specialist herbivores are the typical focus of the study, resulting in assemblages of distantly related parasitoids that makes inferences about drivers of diversification difficult (Antwi et al. 2015; Hood et al. 2015; Leppanen et al. 2014; Nicholls et al. 2018; Stireman et al. 2006). Therefore, studies focusing on closely related parasitoids species are needed to examine patterns of speciation due to ecological divergent selection.

The accurate delimitation of divergent lineages is paramount to speciation studies, as they are often morphologically cryptic. As the cost of generating genomic data has become

increasingly affordable, combined with the advent of multiple programs streamlining the demultiplexing, clustering, and filtering processes, studies utilizing variations of Restriction site Associated DNA sequencing (RADseq) to delimit species and determine drivers of divergence have become more abundant (Bagley et al. 2017; Bernal et al. 2017; de Oca et al. 2017; Eaton & Ree 2013). RADSeq approaches are ideal for detecting population/species level differences and are less susceptible to incomplete lineage sorting and introgression than traditional multigene methods (Andrews et al. 2016), and has been shown to be extremely promising for studies on ecological speciation of herbivorous insects (Bagley et al. 2017; Egan et al. 2015).

Studying the drivers of parasitoid speciation in relation with their hosts is central to understanding their tremendous diversity, while also providing important insights into conservation biology and applied entomology. We chose to address drivers of speciation in the *Lygus-Peristenus* system, which includes species of closely related, economically important herbivores, which are in turn attacked by a group of closely related parasitoid species. The herbivores in this system are the plant bugs in the genus *Lygus* Hahn (Hemiptera: Miridae), which include many species of generalist agricultural pests such as *Lygus lineolaris* Palisot de Beauvois that feeds on a variety of crops. *Lygus* nymphs are indistinguishable morphologically, and thus most literature simply refers to them as *Lygus* species. Inconsistency between the morphological and mitochondrial data *COI* further confounds the accurate identification of *Lygus* (Gwiazdowski et al. 2015), thus rendering most host plant records in the literature dubious. While HAD has been recorded from other Miridae (Hereward et al. 2013), no evidence of HAD has been shown in studies on *Lygus* species despite detection of population level differences (Burange et al. 2012; Zhou et al. 2012). Multiple *Lygus* species often can be found in sympatry, and have one to three generations per year depending on temperature, where southern

populations in warmer climates are multivoltine and more northern populations tend toward univoltinism (Cárcamo et al. 2002; Haye et al. 2013).

Species of *Peristenus* attack nymphal plant bugs as koinobiont endoparasitoids, including *Lygus* species. In the recent revision of the Nearctic *Peristenus pallipes* complex, nine species recognized by Goulet & Mason (2006) were synonymized to three based on morphometrics, mitochondrial DNA (*COI* and *CytB*), and ecological differences (Zhang et al. 2017). This revision also demonstrated a range overlap for *Peristenus dayi* Goulet, *Peristenus mellipes* (Cresson), and *Peristenus howardi* Shaw in southern Alberta (Zhang et al. 2017). As all three *Peristenus* species persist in sympatry in this region, barriers to gene flow preventing hybridization and interbreeding likely exist. These may be ecological isolating mechanisms, such as differences in microhabitat or emergence and reproduction. If so, this would lead to niche partitioning in space or time, which in turn maintains the reproductive barrier between the species. Another possible explanation for the maintenance of three sympatric *Peristenus* species is host preference, but due to the morphological similarity among *Lygus* nymphs, host records of *Peristenus* species in the literature are often listed simply as *Lygus* species. (Goulet & Mason 2006). The Canadian prairies ecosystem is a major agricultural growing region where *Lygus* is an economical pest on several field crops. *Lygus* bugs are closely related and often found in sympatry, HAD could be a driver of population divergence in this system, as genetic cryptic populations could be specializing on certain plants. Therefore, the parasitoids that attack these diverging lineages of *Lygus* could also become sequentially divergent as a result. Literature on the speciation of parasitoids in relation to their hosts is rare, and often limited by few genetic markers (Nicholls et al. 2018; Stireman et al. 2006), or focused on distantly related taxa (Forbes et al. 2010; Hood et al. 2015).

In this study we used a combination of *COI* (mtDNA) and double digest RADSeq (ddRADSeq) (Peterson et al. 2012) to test the drivers of speciation in a group of closely related parasitoids. In particular, we (1) confirm monophyly and delimit species of *Lygus* and their parasitoids *Peristenus*; (2) test for potential host plant associated or temporal differentiation on sympatric species of *Lygus*; and (3) explore for potential sequential HAD or allochronic differentiation as driving forces of speciation on sympatric species of *Peristenus*. This is one of the first studies to address the evolutionary patterns within a tri-trophic system that utilizes host plant, herbivore, and parasitoid using NGS data. Theoretically, herbivore-parasitoid evolutionary histories can provide valuable insights into the evolution of a major portion of biodiversity.

Materials and Methods

Sample Collection and DNA Extraction

In order to obtain *Peristenus* with accurate host records, we sampled early instar nymphal *Lygus* bugs weekly from May to August of 2015 from two sites in Lethbridge, Alberta, as this is the only region in which the range of both *Peristenus mellipes* and *P. howardi* overlaps (Zhang et al. 2017). One additional site in Carman, Manitoba was sampled, where only *P. mellipes* is found. As we were interested in patterns between closely related herbivores and parasitoids, the distantly related *Adelphocoris lineolatus* (Goeze), and by extension their parasitoid *Peristenus dayi* was excluded from this study. While *Lygus* attacks a variety of plants, we chose 3 common host plants: Alfalfa (*Medicago sativa* L.), Yellow Sweetclover (*Melilotus officinalis* (L.)), and Wild Mustard (*Sinapis arvensis* L.) as they were readily accessible and yielded large quantities of nymphs based on pilot studies. We reared nymphs individually in growth chambers (25ºC,

14:10 h L:D photoperiod) using green beans as a food source, and checked daily for parasitoid emergence. When parasitized, the emerged larval parasitoid and host were preserved in 95% EtOH until DNA extraction. Genomic DNA was extracted following the DNeasy Tissue Kit protocol (Qiagen, Valencia, CA, U.S.A.), using a destructive sampling method as the larval parasitoid and host nymphs were unidentifiable morphologically. We quantified the concentration of DNA extracts using Quant-iT High-Sensitivity DNA Assay Kit (Invitrogen, Eugene, OR, USA).

Molecular Data Protocols

We amplified the mitochondrial gene cytochrome oxidase I (*COI*) using universal primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994). Polymerase chain reactions were performed on a Bio-Rad MyCycler thermal cycler (Hercules, CA, U.S.A.), using approximately 1 µg DNA extract, 1X Standard Taq Buffer (10mm Tris–HCl, 50mm KCl, 1.5mm $MgCl_2$, pH 8.3; New England Biolabs, Ipswich, Massachusetts, U.S.A.), 200 µm dNTP (Invitrogen, Carlsbad, California, U.S.A.), 4mm $MgSO_4$, 400 nm of each primer, 1 unit of Taq DNA polymerase (New England Biolabs), and nuclease-free water to a final volume of 25 µL.

We generated *COI* amplicons for both *Lygus* and *Peristenus* with an initial denaturation of 1min at 95°C, followed by 35 cycles of 95°C for 15 s, 49°C for 15 s and 72°C for 45 s, and a final elongation period of 4 min at 72°C. Reaction products were cleaned with Agencourt CleanSEQ magnetic beads (Beckman Coulter Life Sciences, Indianapolis, IN, U.S.A.) and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and the Applied Biosystems 3730xl DNA Analyzer at the

University of Kentucky, Advanced Genetic Technologies Center (UK-AGTC). Contigs were assembled and edited using Geneious version 8.18 (Kearse et al. 2012), and alignment was conducted using MUSCLE using default settings (Edgar 2004). All *COI* sequences were uploaded to GenBank: accession # MG944319 – MG944389.

We used a modified ddRADseq protocol from Peterson et al. (2012) to generate genome wide SNPs for both *Lygus* and *Peristenus*. Based on *in silico* digestion of braconid genomes using SimRAD (Lepais & Weir 2014), the enzyme pair NlaIII and MluCl (NEB, Ipswich, MA, USA) was chosen. Libraries were prepared on 48 individuals grouped by DNA yield, with each sample assigned one of 48 unique 5-base pair (bp) in-line barcode sequences during adapter ligation. Each set of 48 samples was then pooled for automated size selection (216 – 336 bp fragments) on a PippinHT (Sage Science, Beverly, MA, USA). The size-selected samples were then subjected to 12 rounds of high-fidelity PCR amplification (Q5 High-Fidelity DNA Polymerase, NEB) using PCR primers that included one of 12 unique Illumina multiplex read indices. After verifying library quality using high sensitivity DNA kit on TapeStation (Agilent, Santa Clara, CA, USA), libraries were sent to Sanford Burnham Prebys Medical Discovery Institute (Orlando, FL, USA) for sequencing using 2x300bp paired-end reads on a single llumina MiSeq run. All raw fastq files were uploaded onto the NCBI SRA database accession number SRP132595.

We used ipyrad v0.7.23 (Eaton 2014) to process raw sequences, using the following stringent settings to ensure data quality for downstream analyses after parsing out *Lygus* from *Peristenus*: Assembly methods: *de novo*; Minimum depth of reads per within-sample cluster: 10; maximum number of sites in a read which can have a quality score of less than twenty: 4;

clustering threshold: 0.90; minimum number of samples in each across-sample cluster: 10; maximum number of individuals with a shared heterozygous site in an across-sample cluster: 3. These settings were chosen based on multiple test runs with different parameter settings to balance between stringent filtering high quality SNPs calls without losing too much data. All other settings used default values. Additionally, we removed samples with > 80% missing data and suspected haploid *Peristenus* males, which have low heterozygosity.

Phylogenetic Analyses

The best-fitting model of molecular evolution for *COI* was tested using jmodeltest2 (Darriba et al. 2012). The general time-reversible model, with a parameter for invariant sites and rate heterogeneity modelled under a gamma distribution (GTR+I+ Γ), was chosen based on the Bayesian information criterion (BIC). The *COI* sequences were then analyzed using MrBayes v 3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway (Miller et al. 2009). Two independent searches were carried out and four chains run for 2,000,000 generations, sampling every 1000th generation and with a 10% burn-in discarded. The dataset was not partitioned based on nucleotide position as it would limit the amount of data needed for accurate parameter estimation. The phylogenetic trees were visualized in FigTree v1.4.2 (Rambaut 2012) and modified using R package ggtree (Yu et al. 2017). The *Lygus* samples were identified by comparing *COI* sequences with identified adult specimens on the Barcode of Life database (BOLD: http://barcodinglife.org/) that were authoritatively identified by *Lygus* expert Michael D. Schwartz, in cases of ambiguity we chose the identification based on the most common identification (>80%) for each species. Similarly I identified *Peristenus* by comparing the *COI* sequences with samples I used in Zhang et al. (2017).

A maximum likelihood supermatrix approach using the concatenated ddRADSeq SNPs dataset was also conducted with RAxML 8.2.0 (Stamatakis 2006), using the GTR+ Γ model of nucleotide substitution and 1000 bootstrap pseudoreplicates. The resulting trees were visualized and modified in the same manner as the *COI* trees.

Population Genomics Analyses

To determine if there was population structure within clades identified in the phylogenetic analysis, we performed a Bayesian clustering analysis for both *Lygus* and *Peristenus* unlinked SNPs datasets from the ipyrad output stated earlier without prior assignments in Structure v 2.3.4 (Pritchard et al. 2000). We completed ten runs for each population (*K*) up to the maximum number of populations within each clade using 100,000 burnins and 500,000 replicates for each run. The R package pophelper (Francis 2017) was used to visualize the diagrams. The Evanno Δ*K* method (Evanno et al. 2005) was used in Structure Harvester v 0.6.94 (Earl 2012) to determine the most likely value for *K*.

We also created a custom dataset of the SNPs containing only Alberta populations of *P. mellipes* and *P. howardi* in ipyrad using the same settings discussed above. We tested for potential genetic differences under selection between the Alberta populations where the two *Peristenus* species are found in sympatry.

Impacts of locality, host-association, and time of emergence on genetic variation of the three *Lygus* species were tested using AMOVA (Analysis of Molecular Variance) using clustering between localities (for *L. borealis*), host plants (for *L. keltoni* and *L. elisus*), and collecting dates for all three species of *Lygus*. Similarly, AMOVA was used to test for differences between host for both *Peristenus* species, and difference between collection localities

for *P. mellipes*. All AMOVAs were conducted with R packages adegenet (Jombart & Ahmed 2011) and poppr (Kamvar et al. 2014) using the full SNPs dataset as above.

Results

Phylogenetic Analyses

A total of 33 *Lygus* (543 bp) and 37 *Peristenus* (629 bp) *COI* sequences were used for the phylogenetic analysis (Table 4.1). Three monophyletic clades of *Lygus* were identified based on monophyletic clustering with identified specimens available in BOLD: *Lygus borealis* (Kelton), *Lygus keltoni* Schwartz, and *Lygus elisus* Van Duzee (Fig. A4.1). All three species of *Lygus* were collected in Alberta, while only *L. borealis* was collected in Manitoba. Both *L. keltoni* and *L. elisus* were collected from all three host plants, while *L. borealis* was collected exclusively on alfalfa (Table 4.1). *Peristenus mellipes and P. howardi* were reared from these *Lygus* specimens, and both *Peristenus* were recovered as monophyletic clades (Fig. A4.2). *Peristenus mellipes* was reared from all three *Lygus* species and found in both Manitoba and Alberta, while *P. howardi* was reared from *L. borealis* and *L. keltoni* and was found exclusively in Alberta (Table 4.1).

In total, we sequenced a subset of the specimens used in the COI dataset for ddRADSeq. A total of 23 samples of *Lygus* and *Peristenus* each were used, and we obtained an average of ~732,000 reads per individual with an average length of 142bp and average depth of 15x per loci. The final filtered SNPs dataset consists of 14 out of 23 *Lygus* individuals with 1453 parsimonious informative SNPs, and 19 out of 23 *Peristenus* individuals with 18157 parsimonious informative SNPs (Table 4.1). The low number of SNPs recovered from *Lygus* is due to the low DNA quantity as a result of parasitization by *Peristenus*. The topology of the

maximum likelihood trees based on the ddRADSeq data recovered the same clades as the *COI* Bayesian analyses with strong bootstrap support for both all three species of *Lygus* (Fig. 4.1) and both species of *Peristenus* (Fig. 4.2).

Table 4.1. Sampling information for *Lygus* nymphs and the *Peristenus* that emerged from host. GenBank accession number for *COI* and SRA accession number for ddRADSeq provided when available.

Lygus Sample #	ID	GenBank/SRA Accession #	*Peristenus* Sample #	ID	GenBank/SRA Accession #	Locality	Host Plant	Date Collected
YMZ213	*L. borealis*	MG944319/ SAMN08614153	N/A	N/A	N/A	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
N/A	N/A	N/A	YMZ224	*P. mellipes*	MG944353	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ215	*L. borealis*	MG944320	YMZ225	*P. mellipes*	MG944354	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ216	*L. borealis*	MG944321	YMZ226	*P. mellipes*	MG944355/ SAMN08614174	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ217	*L. borealis*	MG944322	YMZ227	*P. mellipes*	MG944356	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ218	*L. borealis*	MG944323/ SAMN08614154	YMZ228	*P. mellipes*	MG944357/SAM N08614175	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ220	*L. borealis*	MG944324/ SAMN08614155	YMZ230	*P. mellipes*	MG944358/ SAMN08614176	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ221	*L. borealis*	MG944325	YMZ231	*P. mellipes*	MG944359/ SAMN08614177	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ222	*L. borealis*	MG944326	YMZ232	*P. mellipes*	MG944360/ SAMN08614178	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ233	*L. keltoni*	MG944327	YMZ243	*P. mellipes*	MG944361/ SAMN08614179	Alberta, Lethbridge, 49.721307, -112.853001	Yellow Clover	30.VI.2015
YMZ234	*L. elisus*	MG944328/ SAMN08614156	YMZ244	*P. mellipes*	MG944362	Alberta, Lethbridge, 49.721307, -112.853001	Yellow Clover	30.VI.2015
YMZ235	*L. keltoni*	MG944329	YMZ245	*P. mellipes*	MG944363/ SAMN08614180	Alberta, Lethbridge, 49.721307, -112.853001	Yellow Clover	30.VI.2015
YMZ236	*L. borealis*	MG944330	YMZ246	*P. mellipes*	MG944364/ SAMN08614181	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015
YMZ237	*L. borealis*	MG944331	YMZ247	*P. howardi*	MG944365/ SAMN08614167	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	08.VIII.2015
N/A	N/A	N/A	YMZ248	*P. howardi*	MG944366	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	08.VIII.2015
YMZ239	*L. elisus*	MG944332/ SAMN08614157	YMZ249	*P. mellipes*	MG944367/ SAMN08614182	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015

Lygus Sample #	ID	GenBank/SRA Accession #	*Peristenus* Sample #	ID	GenBank/SRA Accession #	Locality	Host Plant	Date Collected
N/A	*L. borealis*	N/A	YMZ250	*P. mellipes*	MG944368	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015
YMZ241	*L. borealis*	MG944333/ SAMN08614158	YMZ251	*P. mellipes*	MG944369/ SAMN08614183	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015
N/A	N/A	N/A	YMZ252	*P. mellipes*	MG944370	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015
N/A	N/A	N/A	YMZ263	*P. howardi*	MG944371	Alberta, Lethbridge, 49.721307, -112.853001	Wild Mustard	08.VIII.2015
N/A	N/A	N/A	YMZ264	*P. howardi*	MG944372	Alberta, Lethbridge, 49.721307, -112.853001	Wild Mustard	08.VIII.2015
YMZ255	*L. keltoni*	MG944334/ SAMN08614159	YMZ265	*P. howardi*	MG944373/ SAMN08614168	Alberta, Lethbridge, 49.721307, -112.853001	Wild Mustard	08.VIII.2015
YMZ256	*L. elisus*	MG944335	N/A	N/A	N/A	Alberta, Lethbridge, 49.721307, -112.853001	Wild Mustard	08.VIII.2015
YMZ257	*L. keltoni*	MG944336/ SAMN08614160	YMZ267	*P. howardi*	MG944374/ SAMN08614169	Alberta, Lethbridge, 49.721307, -112.853001	Wild Mustard	08.VIII.2015
YMZ259	*L. keltoni*	MG944337	YMZ269	*P. howardi*	MG944375	Alberta, Lethbridge, 49.721307, -112.853001	Wild Mustard	08.VIII.2015
YMZ260	*L. keltoni*	MG944338/ SAMN08614161	YMZ270	*P. howardi*	MG944376/ SAMN08614169	Alberta, Lethbridge, 49.721307, -112.853001	Alfalfa	08.VIII.2015
YMZ262	*L. elisus*	MG944339	YMZ271	*P. howardi*	MG944377/ SAMN08614170	Alberta, Lethbridge, 49.721307, -112.853001	Alfalfa	08.VIII.2015
YMZ293	*L. borealis*	MG944340	YMZ303	*P. mellipes*	MG944378	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ294	*L. borealis*	MG944341	YMZ304	*P. mellipes*	MG944379	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ295	*L. borealis*	MG944342	N/A	N/A	N/A	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ296	*L. borealis*	MG944343	N/A	N/A	N/A	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ297	*L. borealis*	MG944344	YMZ307	*P. mellipes*	MG944380	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ298	*L. borealis*	MG944345	YMZ308	*P. mellipes*	MG944381	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ299	*L. borealis*	MG944346	N/A	N/A	N/A	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015

Lygus Sample #	ID	GenBank/SRA Accession #	*Peristenus* Sample #	ID	GenBank/SRA Accession #	Locality	Host Plant	Date Collected
YMZ300	*L. borealis*	MG944347	N/A	N/A	N/A	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ301	*L. borealis*	MG944348/ SAMN08614161	YMZ311	*P. mellipes*	MG944382	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	16.VI.2015
YMZ302	*L. borealis*	MG944349	YMZ322	*P. howardi*	MG944383/ SAMN08614184	Manitoba, Carman, 49.500834, -98.023839	Alfalfa	08.VIII.2015
YMZ313	*L. elisus*	SAMN08614163	YMZ323	*P. howardi*	MG944384/ SAMN08614172	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	08.VIII.2015
YMZ314	*L. borealis*	MG944350/ SAMN08614164	YMZ325	*P. howardi*	MG944385/ SAMN08614173	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	08.VIII.2015
YMZ316	*L. elisus*	SAMN08614165	N/A	N/A	N/A	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	08.VIII.2015
YMZ317	*L. borealis*	MG944351	YMZ327	*P. howardi*	MG944386	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	08.VIII.2015
N/A	N/A	N/A	YMZ329	*P. mellipes*	MG944387	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015
N/A	N/A	N/A	YMZ330	*P. mellipes*	MG944388	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	30.VI.2015
YMZ331	*L. keltoni*	MG944352/ SAMN08614166	YMZ332	*P. mellipes*	MG944389/ SAMN08614185	Alberta, Lethbridge, 49.700244, -112.763226	Alfalfa	16.VI.2015

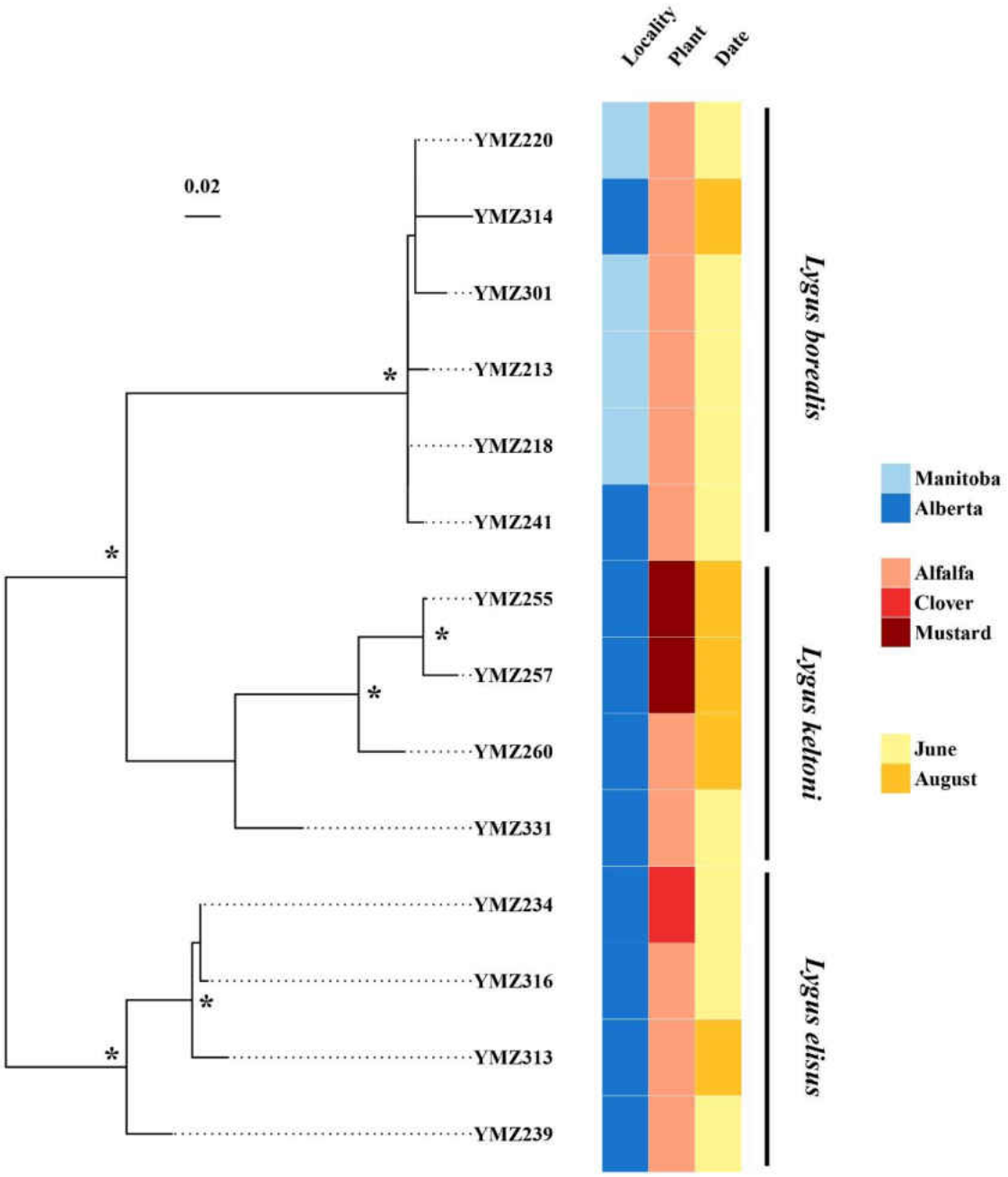

Figure 4.1. Inferred phylogeny of *Lygus* species from the RAxML analysis of the SNPs data. Asterisk indicates bootstrap value of ≥ 90. Sampling locality is colored coded in shades of blue, host plant in shades of red, and collecting date in shades of yellow.

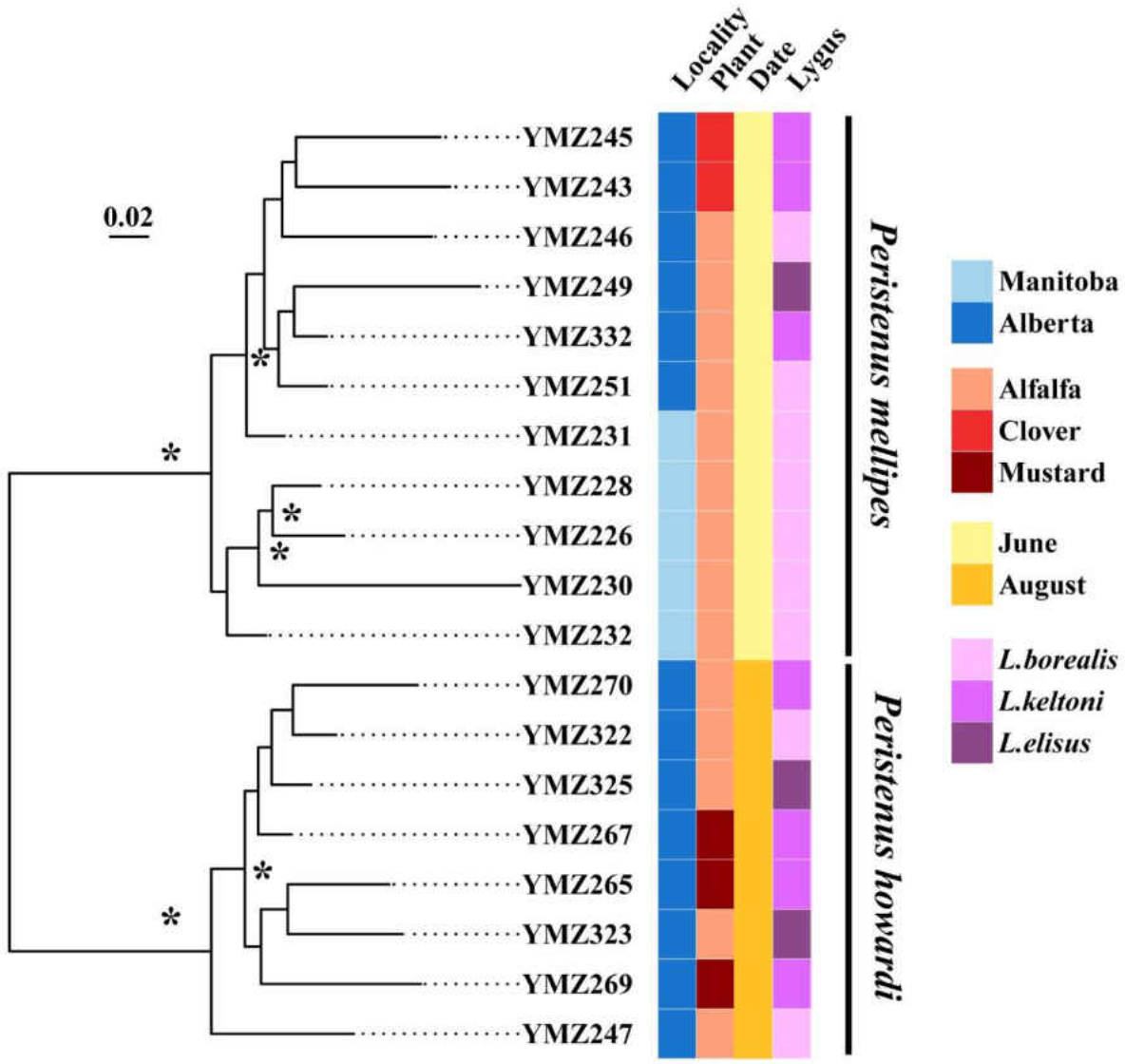

Figure 4.2. Inferred phylogeny of *Peristenus* species from the RAxML analysis of the SNPs data. Asterisk indicates bootstrap value of ≥ 90. Sampling locality is colored coded in shades of blue, host plant in shades of red, collecting date in shades of yellow, and host bug in shades of purple.

Population Genomics Analyses

Using the ΔK approach, Bayesian clustering analyses in STRUCTURE indicated $K = 3$ (Fig. 4.3A) in *Lygus*, which corresponds to the number of species identified by phylogenetic methods (Fig. 4.2). The STRUCTURE results show $K = 3$ among the two *Peristenus* species, as population structure was not found within *P. howardi*, but splits *P. mellipes* into an Alberta-specific population and a Manitoba population (Fig. 4.3B).

No significant genetic differentiation was detected among any of the AMOVA partitions (locality, host plant, collecting date) for the three *Lygus* species (Table 4.2). No differences between host bugs were detected for both species of *Peristenus* (Table 4.3A), but significant genetic differences (p=0.01) was detected among collection localities within *P. mellipes* and explaining 11.77% of the genetic variation (Table 4.3B).

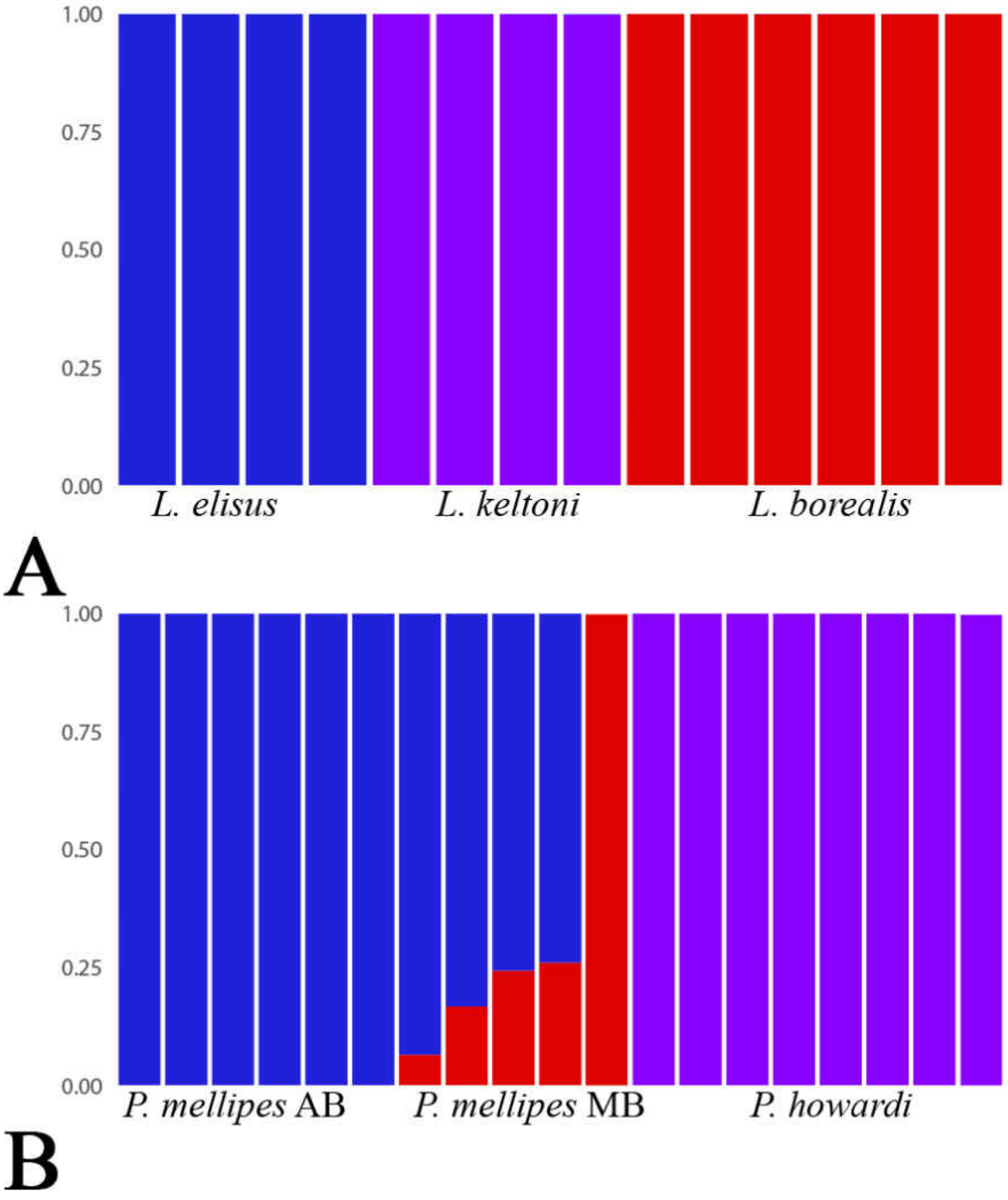

Figure 4.3. STRUCTURE plots of the full SNPs dataset for **(A)** *Lygus* species collected in Manitoba and Alberta, from 3 host plants. The most likely number of partitions was K = 3 (ΔK = 838.45). **(B)** *Peristenus* species reared from the *Lygus* species collected in (A). The most likely number of partitions was K = 3 (ΔK = 7932.06).

Table 4.2. Analysis of Molecular Variance (AMOVA) using clustering between (a) localities, (b) host plants, and (c) collecting dates for all three species of *Lygus* used in this study.

Taxon Assessed	Source of Variation	*df*	Variance component	% total variation	*Φ*-statistics	*p*-value
a) Between Localities						
L. borealis	Between localities	1	-1.55	-5.95	-0.73	0.95
	Among samples within localities	4	-17.74	-67.79	-0.64	1.00
	Within samples	6	46.48	173.74	-0.06	1.00
b) Between Host Plants						
L. keltoni	Among plants	1	1.09	3.01	-0.73	0.37
	Among samples within plants	2	-27.61	-76.09	-0.78	0.89
	Within samples	4	62.79	173.08	10.03	0.98
L. elisus	Among plants	1	0.20	0.64	-0.87	0.71
	Among samples within plants	3	-27.90	-87.95	-0.89	0.93
	Within samples	4	59.43	187.31	0.01	1.00
c) Between Collection Dates						
L. borealis	Among dates	1	-2.21	-8.69	-0.78	0.87
	Among samples within dates	4	-17.84	-70.15	-0.64	0.99
	Within samples	6	45.48	178.83	-0.09	1.00
L. keltoni	Among dates	1	1.16	3.17	-0.72	0.45
	Among samples within dates	2	-27.46	-75.24	-0.78	0.96
	Within samples	4	62.79	172.07	0.03	1.00
L. elisus	Among dates	1	-0.87	-2.78	-0.91	1.00
	Among samples within dates	2	-27.37	-87.74	-0.85	1.00
	Within samples	4	59.43	190.52	-0.03	1.00

Table 4.3. (A) Analysis of Molecular Variance (AMOVA) using clustering between different localities and different host bugs for both species of *Peristenus* used in this study. **(B)** Hierarchical AMOVA of collection localities grouped within host bug, and host bugs grouped within localities for *Peristenus mellipes*. Significant *p*-values are bolded.

Taxon Assessed	Source of Variation	*df*	Variance component	% total variation	*Φ*-statistics	*p*-value
a) Between Host Bugs						
P. mellipes	Between bugs	2	110.19	12.42	0.44	0.11
	Among samples within bugs	8	282.52	31.84	0.36	**0.01**
	Within samples	11	494.54	55.74	0.12	**0.01**
P. howardi	Between bugs	2	-12.10	-2.44	0.37	0.66
	Among samples within bugs	5	193.81	39.03	0.38	**0.02**
	Within samples	8	314.86	63.41	-0.02	**0.01**
b) Between Localities	Between localities	1	104.39	11.77	0.44	**0.01**
P. mellipes	Among samples within localities	9	287.69	32.45	0.37	**0.02**
	Within samples	11	494.54	55.78	0.12	**0.01**

Discussion

Identification of Lygus and Peristenus Using Molecular Data

The accurate identification of *Lygus* has been problematic in the past, because of the inconsistency of morphological differences of nymphs and *COI* data (Gwiazdowski et al. 2015). The *Lygus* species included in this study, *L. borealis*, *L. elisus*, and *L. keltoni*, were often misidentified even by experts because of their adult phenotypic variability. This taxonomic confusion has made previous host records in this group unreliable. Using *COI* and SNPs, we confirmed the identity of the *Lygus* nymphs used in this study and established accurate host records for the parasitoids. Taxonomic revision of *Lygus* is needed, as current morphological characters without the aid of molecular tools is unreliable, and we advise caution when using publicly available databases such as GenBank and BOLD as misidentifications are common despite expert identification. The identification of *Peristenus mellipes* and *P. howardi* using both *COI* and SNPs is consistent with Zhang et al. (2017).

Lack of HAD and Allochrony Within Lygus Species

Based on the result of our phylogenetic tree (Fig. 4.1) and AMOVA analyses (Table 4.2), it is unlikely that *Lygus* species evolved through host-associated differentiation in the Canadian prairies. The three species of *Lygus* are all generalist herbivores feeding indiscriminately on available food sources, as no host-plant-specific lineages are found within each species (Fig. 4.1, Table 4.2). While both *L. elisus* and *L. keltoni* were found on all three host plants sampled in this study, *L. borealis* were only found from alfalfa. The apparently narrow host range of *L. borealis* could be a byproduct of our sampling, as they have been collected from other host plants such as canola (*Brassica* spp.) in other studies (Cárcamo et al. 2002; Otani & Cárcamo 2011). These

results show that *Lygus* species are truly generalists, feeding on a variety of host plants but do not exhibit HAD. This lack of HAD is consistent with studies of other *Lygus* species such as *L. lineolaris* (Burange et al. 2012) and *L. hesperus* (Zhou et al. 2012) despite detection of population level differences, indicating factors other than HAD likely drove their evolution.

Allochrony but No HAD Within Peristenus Species

The host choice between the two species of *Peristenus* were not significantly different in the hierarchical AMOVAs (Table 4.3A) and the majority of variation occurred within samples, suggesting factors other than hosts are likely driving the bulk of the genetic variation. This is further corroborated by the lack of host-specific lineages within each of the *Peristenus* species (Fig. 4.2). Unlike their herbivore hosts, the two *Peristenus* species exhibit temporal differentiation in Alberta, where both species occur (Fig. 4.2). Both species appear to be attacking all available hosts upon emergence, with *P. mellipes* appearing early in June and attacking the first generation of *Lygus*, while *P. howardi* emerging later in August and attacking the second *Lygus* generation. This temporal separation could be the result of selection for niche partitioning to avoid direct competition, as both *Peristenus* species are ecological competitors that occur in the same geographical and host ranges. Another possible explanation is the presence of this temporal heterogeneity could pre-date the contact of the two *Peristenus* species, however this is unlikely as both species collected outside of this contact zone in Alberta are not bound by this strict temporal separation (Zhang et al. 2017). Our findings are consistent with Fernández et al. (unpublished data), who found *P. mellipes* occurs early in the season between late May to late July, and *P. howardi* in late June to late August. In addition, emergence times of *P. mellipes* were on average 13 days earlier than *P. howardi* in laboratory trials (Fernández *et al.*

unpublished data). It is unknown how common parasitoids exhibit allochronic speciation, but in theory divergent ecological selection in parasitoids can have similar genome-wide effects as herbivorous insects, especially if considerable standing genomic variation is already present (Egan et al. 2015; Michel et al. 2010).

Interestingly, both STRUCTURE (Fig. 4.3B) and AMOVA (Table 4.3B) detected population structures within *P. mellipes* that splits the Manitoba population from Alberta (11.77% variation, $p = 0.01$). However, the majority of the genetic variation is still within samples of each site (55.78% variation, $p = 0.01$), suggesting other factors are responsible for the genetic variation observed. Additionally, no host-associated patterns were observed as Manitoba samples only consisted of wasps reared from *L. borealis* feeding on alfalfa (Table 4.3). The Manitoba *P. mellipes* has only one generation per year despite the absence of *P. howardi*, which could be the result of their host phenology as Manitoba has a shorter summer than Alberta, thus only allowing for the development of one full generation of *Lygus* (Haye et al. 2013). While *P. mellipes* were only collected from Canadian prairies in this study, previous work (Zhang et al. 2017) and historical records have shown that there are two generations of *Lygus* and *P. mellipes* in warmer regions such as Ontario (Goulet & Mason 2006). *Peristenus mellipes* was previously separated as four distinct species (*P. mellipes*, *P. pseudopallipes*, *P. carcamoi*, and *P. otaniae*) based on emergence time differences (Goulet & Mason 2006; Lim & Stewart 1976; Loan 1970), but they were synonymized under *P. mellipes* because of the lack of mtDNA and morphometric differences (Zhang et al. 2017). This study is limited in terms of host plant breadth and sampling across the entire range of both *Peristenus* species, thus future studies should include additional populations from multiple host plants that cover the entire range of *P. mellipes* to determine the degree of gene flow between the eastern populations and Manitoba. The third species within the

Nearctic *Peristenus pallipes* complex is *P. dayi*, which emerges earlier than *P. mellipes*, with peak activity late May to early June. *Peristenus dayi* attacks *A. lineolatus* rather than *Lygus* spp. (Goulet & Mason 2006; Zhang et al. 2017). While they were not the focus of the current study, the partial host and temporal separation between the three closely related *Peristenus* species presents an interesting hypothesis to their evolutionary history and could be tested using similar methods as our current study.

The specialization of *Peristenus* on different generations of *Lygus* may have caused differences in breeding time which then led to temporal assortative mating and limited gene flow, essentially equates to allopatric populations separated by temporal differentiation (Taylor & Friesen 2017). Differences in breeding time can therefore be interpreted as an alternate to spatial differentiation, or as a type of ecological differentiation that warrants further attention as examples in literature remains sparse (Taylor & Friesen 2017). Studies on the prevalence of parasitoids feeding on generalist herbivores and the factors that drive their speciation would also yield interesting insights.

Conclusion

Using mitochondrial DNA and genome-wide SNPs, our comparative analysis of genetic differentiation between the two sister *Peristenus* species attacking multiple *Lygus* hosts revealed temporal speciation rather than host-associated differentiation. Allochrony likely played a vital role in the speciation process of *Peristenus*, whether it is acting alone or in concert with host preferences or other pre- or post-zygotic barriers to gene flow. This is one of the first studies to demonstrate the potential of genomic data in resolving the tri-trophic evolutionary relationships

between plant, herbivore, and parasitoids. This study also demonstrates the importance of systematics to studies of parasitoid speciation, particularly careful delimitation of cryptic species, host rearing to obtain accurate records, and genomic scale data for examining any population level differences among closely related taxa.

Given the results of our study, the *Lygus-Peristenus* system can also be added to the growing body of literature on the importance of temporal separation as a driving force for ecological speciation and its effect on the evolution of the rich diversity of life. The origin and maintenance of the reproductive barrier between the closely related parasitoid species is likely a product of adaptation to their host phenology. As many phytophagous insects and their parasitoids systems are well studied as a result of their agricultural and economical importance, large, collaborative, genomic-scale parallel studies across multiple taxa at different stages of species continuum could yield valuable insights into the prevalence and impact of allochrony in ecological speciation.

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CHAPTER FIVE: CONCLUSION AND SIGNIFICANCE

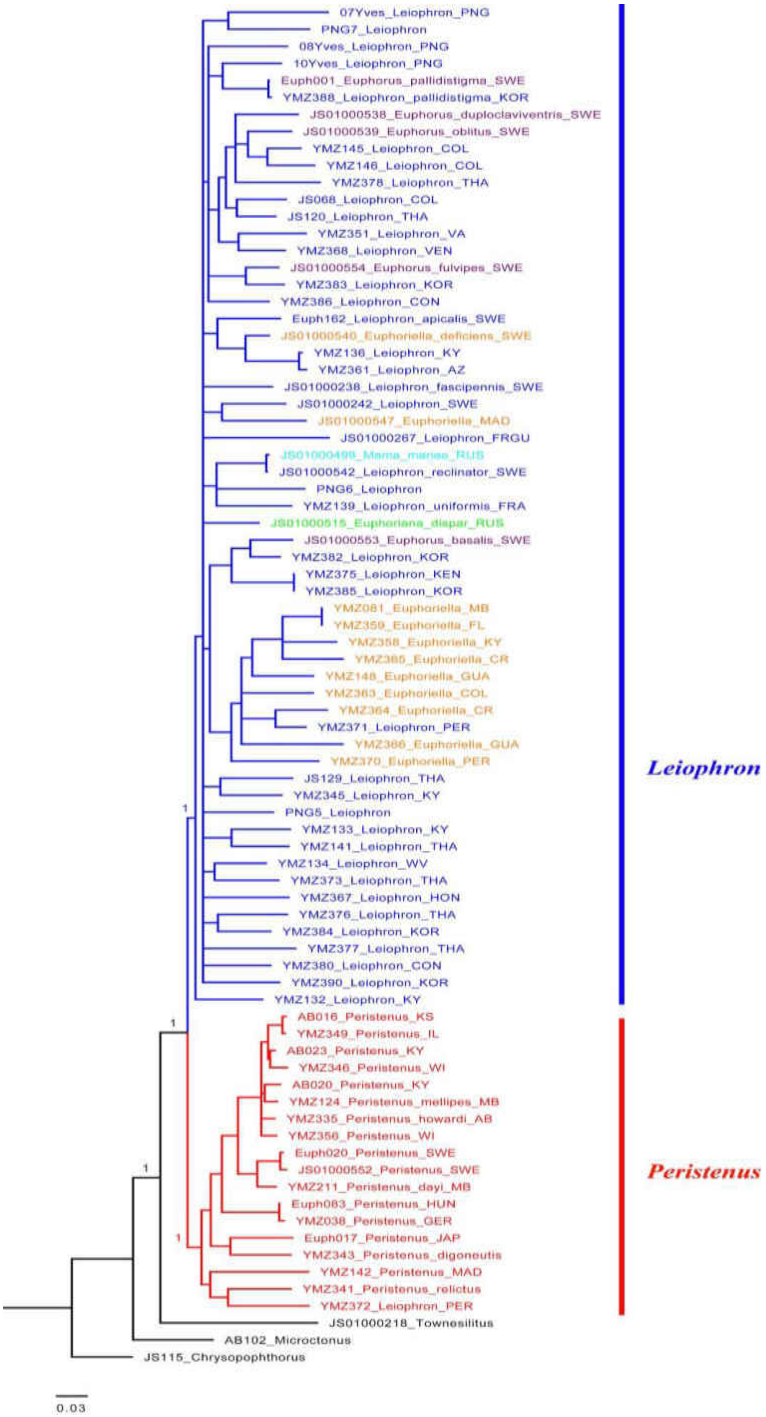
Parasitoids wasps represent one of the largest groups of understudied organisms on earth, making them ideal model organisms for the refinement of evolutionary theories such as speciation. Understanding the evolution of parasitoid wasps with their hosts is also essential for applied entomological research, as inadequate understandings of the taxonomy, phylogenetic relationships, and evolutionary history with their hosts will result in mismatched candidates for biological control agents. Many parasitoid species also play critical roles in food chains, and their complex, specialized life history strategies are of conservational concern in a time of rapid habitat loss and climate change. As the field of phylogenetics and evolutionary biology rapidly shifts into the era of genomic data, we are offered an unprecedented opportunity to gain insights into the complex evolution of parasitoid wasps using large, genome-wide datasets. However, despite the influx of genomic data, the importance of ecological and morphological data remains relevant, if not more so than ever. Only by examining genomic data in the context of ecological or morphological information, will it be possible to tease apart the evolutionary history of parasitoid wasps.

Using the euphorine braconids as a model, I have demonstrated the need for combining ecological data with morphological and genetic/genomic data to examine their evolutionary history at different taxonomic scales. From Chapter 2 I was able to elucidate the phylogenetic relationships within the tribe Euphorini using three genes in combination with morphology. The monotypic genus *Mama* and the subgenera within *Leiophron s.l.* were synonymized, and the generic concepts of the *Peristenus* and *Leiophron* were updated in light of these results. The robust phylogeny resolved the long history of taxonomic uncertainty associated with Euphorini,

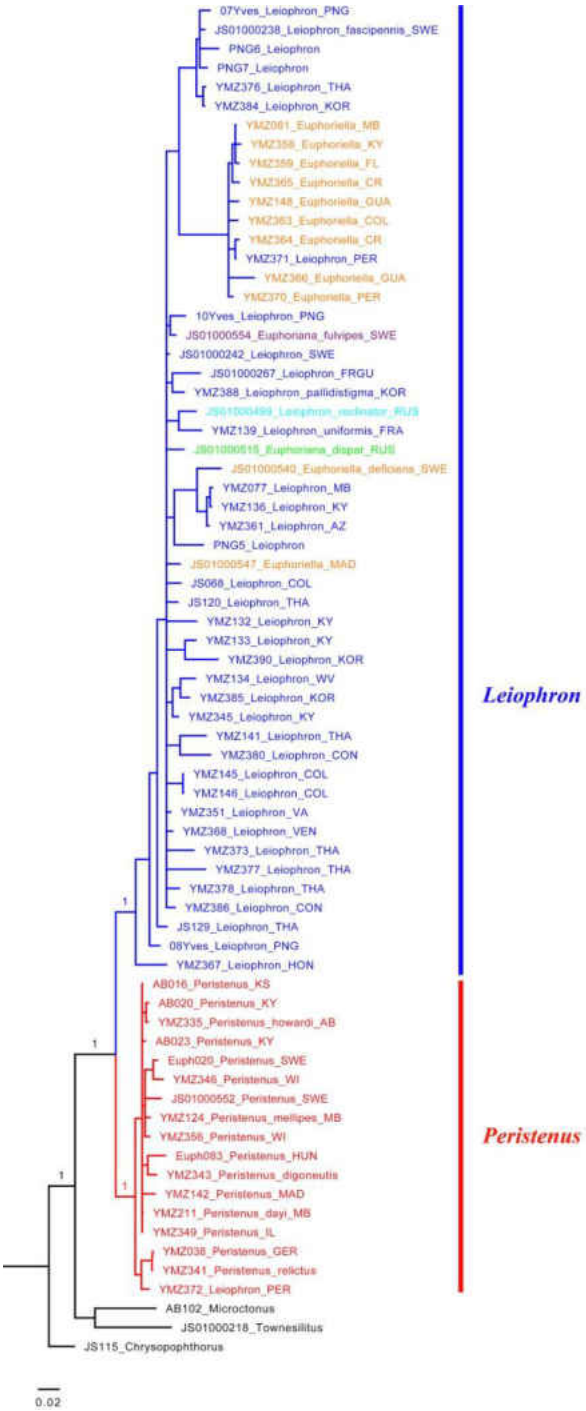
and new morphological characters will aid in accurate identification of these economically important wasps. In Chapter 3 I used a combination of morphological, molecular, and ecological data to resolve the Nearctic *Peristenus pallipes* complex. This integrative taxonomic approach resulted in multiple synonymies, reducing the number of species down from nine to three. This update clarifies the taxonomic confusion surrounded the Nearctic *Peristenus pallipes* group and provide a dichotomous key to aid in the accurate identification of this group. Additionally, I highlight the importance of foreign biocontrol agents displacing native species, and the need for collaboration between taxonomists and applied entomologists. Finally, in Chapter 4 I used ddRADSeq to generate SNPs for both *Peristenus* along with their *Lygus* bug hosts, and examined their evolutionary histories in relation to host-associated or temporal separation. The two *Peristenus* examined in this final chapter appears to have evolved as a result of temporal separation, while their hosts did not exhibit host-associated or temporal separation. This difference between host and parasitoid evolutionary history provides an interesting example of ecological speciation for future studies on speciation, at the same time raises concerns about incorporating evolutionary history of parasitoids in applied studies ranging to biocontrol and conservation of parasitoids.

Taken as a whole, my dissertation provided updated phylogenies of *Peristenus* at the tribal, generic, and species level, as well as population genomic level insights into their evolutionary history with their hosts. These results advance our understanding of Braconidae evolution and highlight the need for more targeted phylogenomics studies on parasitic wasps. My contribution to understanding this tiny portion on the tree of life will hopefully join many other studies at the similar scales, and ultimately help in our ongoing pursuit of understanding the tree of life.

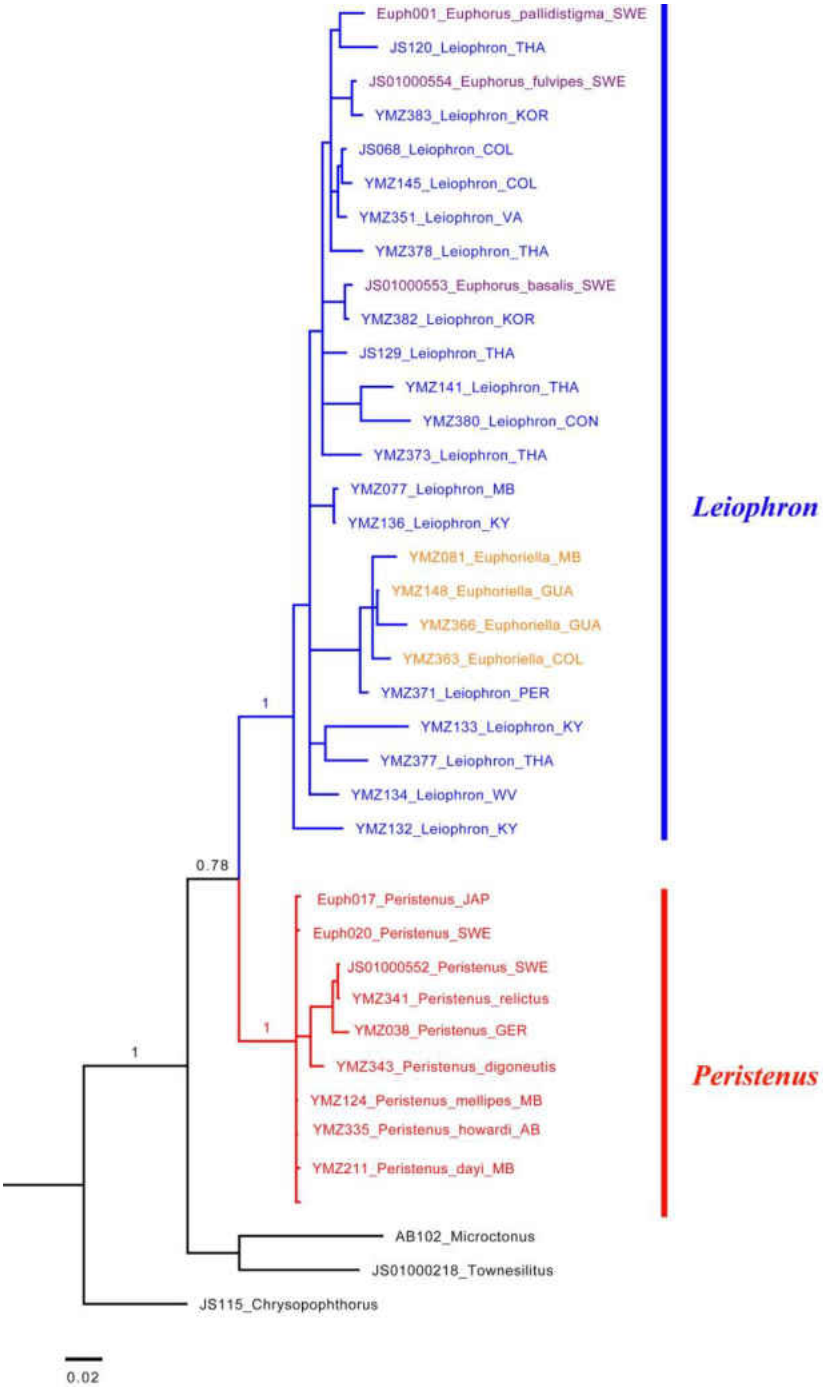
APPENDIX A: CHAPTER TWO SUPPORTING INFORMATION



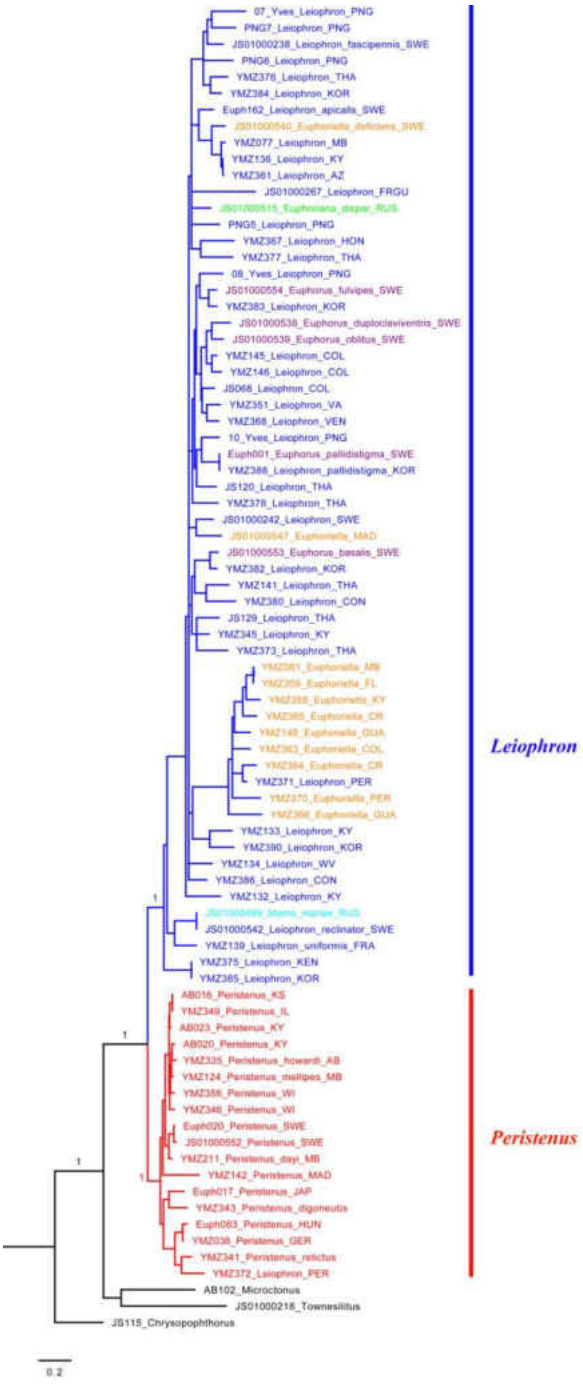
Supplementary Figure 2.1. *COI* gene tree for MrBayes. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support (≥ 0.98 posterior probability).



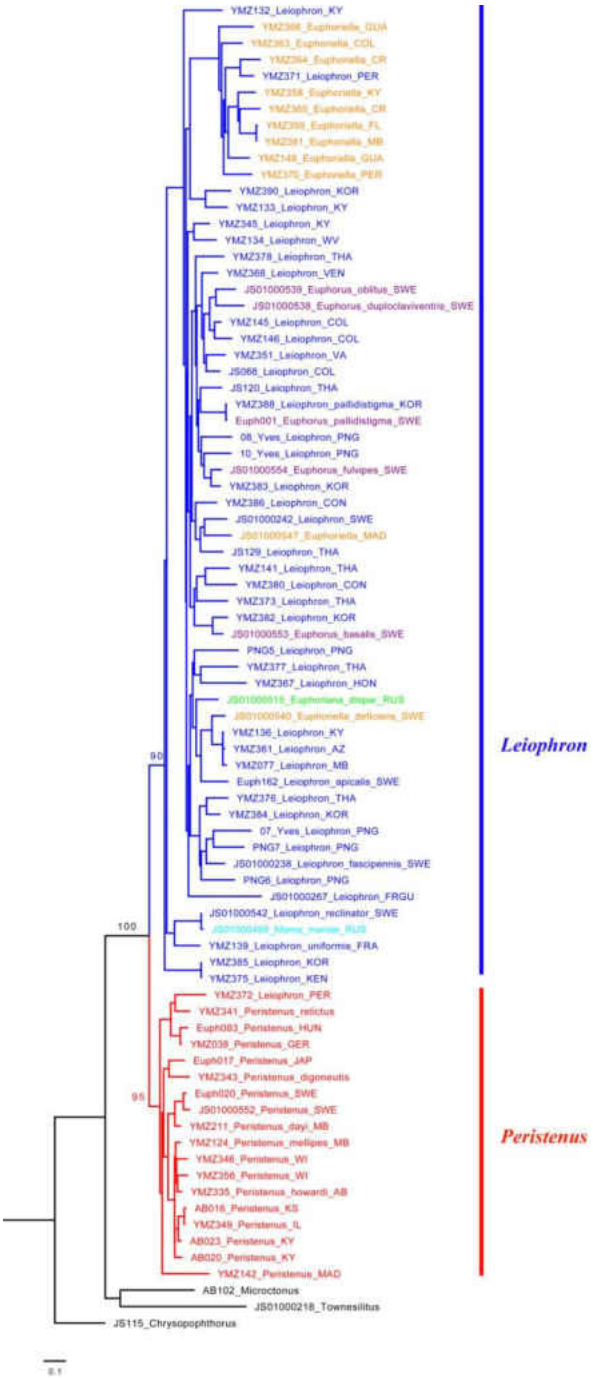
Supplementary Figure 2.2. *28S* gene tree for MrBayes. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support (≥ 0.98 posterior probability).



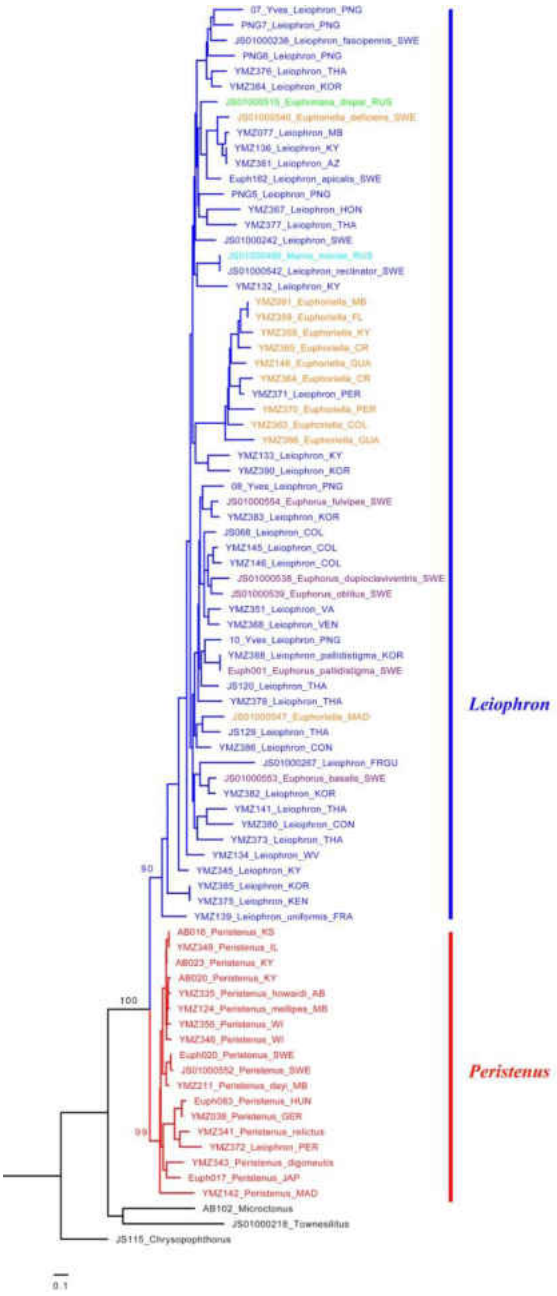
Supplementary Figure 2.3. *CAD* gene tree for MrBayes. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support (≥ 0.98 posterior probability).



Supplementary Figure 2.4. Concatenated gene trees for MrBayes. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support (≥ 0.98 posterior probability).



Supplementary Figure 2.5. Concatenated gene trees for RAxML. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support (≥90 for bootstrap support).



Supplementary Figure 2.6. Concatenated gene trees for IQ-Tree. *Peristenus* is colored red, and *Leiophron* is colored in blue, with subgenera within *Leiophron* shown in different colors (*Leiophron sensu stricto* in blue, *Euphorus* in purple, *Euphoriana* in green, *Euphoriella* in orange, and *Mama* in brown). Asterisks indicate strong nodal support (≥90 for ultrafast bootstrap support).

Supplementary Table 2.1. Detailed collection locality information for all specimens used in this study.

Taxon Label	Locality Label (verbatim)
07_Yves_Leiophron_PNG	Papua-New-Guinea, Province Madang, Mount Wilhelm 200m (-5.739897, 145.3297), 31.X—01.XI.2012, leg Dilu, Ray, Novotny, Leponce, Plot 1, understorey; Malaise - MAL-MW0200A-07/16-d07
08_Yves_Leiophron_PNG	Papua-New-Guinea, Province Madang, Mount Wilhelm 1700m (-5.759269, 145.2356), 27—28.X.2012, leg Valeba, Tulei, Novotny, Leponce, Plot 4, understorey; Malaise - MAL-MW1700A-03/16-d03
10_Yves_Leiophron_PNG	Papua-New-Guinea, Province Madang, Mount Wilhelm 2700m (-5.814968, 145.1580), 18—19.X.2012, leg Kua, Yalang, Novotny, Leponce, Plot 4, understorey; Malaise - MAL-MW2700D-03/16-d03
AB016_Peristenus_KS	USA, Kansas, Riley Co. Konza Prairie Biol. Sta. 4B 39°06.65'N 96°35.75'W MT 25—29.X.2001, Zolnerowich, Kula, Brown
AB020_Peristenus_KY	USA, Kentucky, Harrison Co Silverlake Farm, Savanna 38°19.553'N 84°21.428'W MT 3 Hickory Edge [HI#7] 28.IV—5.V.2004, Hym. Inst.
AB023_Peristenus_KS	USA, Kansas, Riley Co. Konza Prairie Biol. Sta. Kings 39°06.20'N 96°35.77'W MT [HI#87] 1—4.V.2001, Zolnerowich, Kula, Brown
Euph_001_Euphorus_pallidistigma_SWE	Sweden, Öland Gårdby, 56°35'05.0N 16°36'54.6E 5.VII.2014, C.Hansson
Euph_017_Peristenus_JAP	Japan, Hyôgo Pref, Kobe-shi, Tanigami. 15.V—1.VI.2011 Malaise trap field. 34.7589 N, 135.1722 E. Leg. J. Stigenberg, H. Vårdal
Euph_020_Peristenus_SWE	Sweden, Skåne, Billebjär, 55.41188 13.19200, 16.V.2014, C.Hansson
Euph_083_Peristenus_HUN	Hungary, Köszeg NW outskirts of town, 21—27.VI.2010, MT in garden.
Euph_162_Leiophron_apicalis_SWE	Sweden, Öland, Gamla Skogsby (Kalkstad) mixed decidious forest, 27.V—27.VI.2014 Malaise trap, M. & C. Jaschhof. Jaschhof catalog #63/2014
JS01000238_Leiophron_fascipennis_SWE	Sweden, Sm, Nybro kommun, Bäckebo, Grytsjöns naturreservat Old moisty haymaking meadow in forest edge N6311678 E1517066 (=TrapID 1001), 02.VII—12.VII.2005 (=coll. event ID 1332)
JS01000242_Leiophron_SWE	Sweden, Öl, Mörbylånga kommun, Frösslunda alvar, north eastern part, alvar pasture. N56°32.847´ E16°34.635´ (=TrapID 20) 05.VII—02.VIII.2005 (=coll.event ID 1498)

Taxon Label	Locality Label (verbatim)
JS01000267_Leiophron_FRGU	French Guiana, Montagne des Chevaux, 15.VII—8.VIII.2011
JS01000499_Mama_mariae_RUS	Russia, Primorskiy kray 10 km SE Partizansk, Novitskoe, forest, glades. 3-4.VIII.2010 Belokobylskij
JS01000515_Euphoriana_dispar_RUS	Russia, Primorskiy kray 10 km E Spassk-Dal'niy, 30.VI.2010 forest, glades. S. Belokobylskij
JS01000538_Euphorus_duploclaviventris_SWE	Sweden, Sm, Gränna kommun, Lönnemålen. Next to old cellar in Norway spruce forest w. big harvested ashes. N58°02.935´ E14°34.382´ (=Trap ID 17) 31.V—15.VI.2005 (=coll. event ID 1514)
JS01000539_Euphorus_oblitus_SWE	Sweden, Bl, Ronneby kommun, Tromtö, Tromtö nabb. Beech and oak forest. N56°08.944´ E15°28.801´ (=TrapID 23) 20.V—03.VI.2004 (=coll. event ID 449)
JS01000540_Euphoriella_deficiens_SWE	Sweden, Sk. Malmö. Limhamns Kalkbrott, Malaise trap 2-"planen", 26.VI—8.VII. 2009, leg. B.W.Svensson & Co
JS01000542_Leiophron_reclinator_SWE	Sweden, Sk. Malmö. Limhamns Kalkbrott, Malaise trap 1-"grafitti", 26.VI—8.VII. 2009, leg. B.W.Svensson & Co
JS01000547_Euphoriella_MAD	Madagascar, Finanrantsoa 16.XII.2011—1.I.2012
JS01000552_Peristenus_SWE	Sweden, Sdm, Trosa kommun, Askö naturreservat. Malaise Trap N58°48,420", E17°40.437'. -1 moh. 14.VI—29.VI.2011. Loc.030-02 Leg.B.E. Bengtsson
JS01000553_Euphorus_basalis_SWE	Sweden, Sdm, Trosa kommun, Askö naturreservat. Malaise Trap N58°48,420", E17°40.437'. -1 moh. 14.VI—29.VI.2011. Loc.030-02 Leg.B.E. Bengtsson
JS01000554_Euphorus_fulvipes_SWE	Sweden, Sdm, Trosa kommun, Askö naturreservat. Malaise Trap N58°48,420", E17°40.437'. -1 moh. 14.VI—29.VI.2011. Loc.030-02 Leg.B.E. Bengtsson
JS068_Leiophron_COL	Colombia, Boyaca, Iguaque M. 3533
JS120_Leiophron_THA	Thailand, Trang Pr., 140m. 20—27.I.2005
JS129_Leiophron_THA	Thailand, Trang Pr., 140m. 20—27.I.2005
PNG_5_Leiophron	Papua-New-Guinea, Province Madang, Mount Wilhelm 3200m (-5.806944, 145.0721), 20—21.X.2012, leg Dahl, Kaupa, Novotny, Leponce, Plot 3, understorey; Malaise - MAL-MW3200C-05/16-d05; P3374
PNG_6_Leiophron	Papua-New-Guinea, Province Madang, Wanang 3 station (-5.22767, 145.0797) 175m, 18—19.XI,2012, leg Basset, Plot 3, understorey; Malaise - MAL-WAN03-D01 P4932
PNG_7_Leiophron	Papua-New-Guinea, Province Madang, Wanang 3 station (-5.22767, 145.0797) 175m, 02—

Taxon Label	Locality Label (verbatim)
	03.XII.2012, leg Basset, Plot 1, understorey; Malaise - MAL-WAN01-D15 P4914
YMZ038_Peristenus_GER	Germany, Schleswig-Holstein, 11.VIII.2001
YMZ077_Leiophron_uniformis_MB	Canada, Manitoba, Carman, 17.VI.2013, Y, Miles Zhang
YMZ081_Euphoriella_MB	Canada, Manitoba, Carman, 17.VI.2013, Y, Miles Zhang
YMZ124_Peristenus_mellipes_MB	Canada, Manitoba, Beaudry Provincial Park, 10.X.2014
YMZ132_Leiophron_KY	USA, Kentucky, Franklin Co. Cove Springs Park 38 13.237N 84 51.414W MT2 wooded clearing [HI#235] 14—21.VI.2005 K. Pitz
YMZ133_Leiophron_KY	USA, Kentucky, Franklin Co. Lexington: Tee It Up Golf 37 58'39"N 84 24'59"W MT: malaise trap [HI#200] 10—17.IX.2004, B. Sharanowski
YMZ134_Leiophron_WV	USA, West Virginia, Hardy Co. 3mi NE Mathias 38 55'N 78 49'W 28.V—4.VII.2004, MT, David R. Smith
YMZ136_Leiophron_KY	USA, Kentucky, Fayette Co. Lexingon 1118 Slashes Rd 83 29'07"w 38 01'45"N MT: M. Sharkey X.2003
YMZ139_Leiophron_uniformis_FRA	France, Herault Baillarguet CSIRO lab 43 41'12"N 3 52'24'E 3—15.V.1993 P.G. Mason champ sauvage, MT
YMZ141_Leiophron_THA	Thailand, Chiang Rai Prov Doi Luang National Park Namtok Pu Kaeng, 540m 19 26'N 99 42'E MT: [HI#62] 16.III.2002 Coll: Mercury Vapour Lamp
YMZ142_Peristenus_MAD	Madagascar, Fianarantsoa Prov Parc Nat, Ranomafana, radio tower@ forest edge 22—26 XI.2001, ele 1130m Calif. Aca. Sci. 21 15.05'S 47 24. 43'E MA-02-09B-04 Caslot 014023, MT, mixed tropical forest Coll: R. Harin' Hala
YMZ145_Leiophron_COL	Colombia, Cundinamarca PNN Sumapaz Bocatoma. Cerro el zapato 4 14'N 74 12'W 3560m MT 18.XI—4.XII.2002 A. Patino Leg,M.3443
YMZ146_Leiophron_COL	Colombia, Cundinamarca PNN Sumapaz Bocatoma. Cerro el zapato 4 14'N 74 12'W 3560m MT 18.XI—4.XII.2002 A. Patino Leg,M.3443
YMZ148_Euphoriella_GUA	Guatemala, Suchitepeque Finca Moca Grande hill behindlake, MT 23—24.II.1995, D. Quintero A.
YMZ211_Peristenus_dayi_MB	Canada, Manitoba, Nopiming Provincial Park, 06/2012, Sharanowski Lab
YMZ335_Peristenus_howardi_AB	Canada, Lethbridge, Peenaquim Park, 14.VII.2011

Taxon Label	Locality Label (verbatim)
YMZ341_Peristenus_relictus_LAB_COLONY	USA, New Jersey, Philip Alampi Beneficial Insect Lab, New Jersey Department of Agriculture, 09.VI.2016
YMZ343_Peristenus_digoneutis_LAB_COLONY	USA, New Jersey, Philip Alampi Beneficial Insect Lab, New Jersey Department of Agriculture, 09.VI.2016
YMZ345_Leiophron_KY	USA, Kentucky, Herndon Farm 38 33 25N 084 59 35W MT 6 Shed, f-c interface 5.VIII—17.VIII 2009, 151m Hym Institute
YMZ346_Peristenus_WI	USA, Wisconsin, La Crosse Co. nr West Salem 43 54 22.22N 91 10 52 W 21—30.V.2010, alt 387 MT A.M. Shorter
YMZ348_Leiophron_IL	USA, Illinois, Lee Co, Richardson Wildlife Foundation 41 32 26.91N 89 11 12.79W 20—29.VII.2010. 252m Terry Moyer
YMZ349_Peristenus_IL	USA, Illinois, Lee Co, Richardson Wildlife Foundation 41 32 26.91N 89 11 12.79W 20—29.VII.2010. 252m Terry Moyer
YMZ351_Leiophron_VA	USA, Virginia, Hanover Co. 2.39k NW Vontay N 37.765172 W 77.775934 MT 28.V—11.VI.2011, AV Evans, JC Ludwing
YMZ356_Peristenus_WI	USA, Wisconsin, La Crosse Co. nr West Salem 43 54 22.22N 91 10 52 W 11—21.VI.2010. alt 387 MT A.M. Shorter
YMZ358_Euphoriella_KY	USA, Kentucky, Franklin Co. Cove Springs Park 38°13.178'N 84°51.325W MT 1: Floodplain [HI#234] 14—21.VI.2005. K. Pitz
YMZ359_Euphoriella_FL	USA, Florida, Alachua Co. Gainesville, AEI 29°35'53.6"N 82°21'54.8"W IV.2005, MT D.B. Wahl
YMZ361_Leiophron_AZ	USA, Arizona, Cochise Co. Bishee, 1429 Franklin Street, Malise in dry wash, 1585m 31.4038°N 109.9262°W 18—28.V.2015 AS Menke
YMZ363_Euphoriella_COL	Colombia, Valle del Cauca PNN Farallones de Cali Cgto. La Meseta 3°34'N 76°40'W 2080m Malaise 10—25.II.2004 S. Sarria & M. Losso Leg. 4555
YMZ364_Euphoriella_CR	Costa Rica, Prov. Heredia 6km ENE Vara Blanca 10°11'N 84°07'W 2000m 20/M/18/038, 10.III.2002 INBio-OET-transect
YMZ365_Euphoriella_CR	Costa Rica, Prov. Heredia 6km ENE Vara Blanca 10°11'N 84°07'W 2000m 20/M/12/072, 9.IV.2002 INBio-OET-ALAS-transect
YMZ366_Euphoriella_GUA	Guatemala, Peten Parq. Nac. Tikal 17.24030 -89.62207 6m-270m 22.V.2009. LLAMA#Wa-B-05-2-01

Taxon Label	Locality Label (verbatim)
YMZ367_Leiophron_HON	Honduras, Atlantida 7km SSW Tela 15.72417 -87.45187 150m-190m 15.VI.2010, LLAMA#Wa-C-08-2-all
YMZ368_Leiophron_VEN	Venezuela, Aragua Rancho Grande 1140m 1—6.III.1995. R.W.Brooks. FIT
YMZ370_Euphoriella_PER	Peru, Wayqecha Oso S13°11.370 W71°35.074 16—28.VII.2014 Sharanowski Lab
YMZ371_Leiophron_PER	Peru, Wayqecha Oso S13°11.370 W71°35.074 16—28.VII.2014 Sharanowski Lab
YMZ372_Peristenus_PER	Peru, Wayqecha Oso S13°11.370 W71°35.074 16—28.VII.2014 Sharanowski Lab
YMZ373_Leiophron_THA	Thailand, Petchaburi Kaeng Krachan NP Pa La-U/Haui Palao Forest Unit 3 12°32.149'N 99°28.265'E Malaise Trap 18—25.I.2009 Thongbai leg. T4566
YMZ375_Leiophron_KEN	Kenya, Eastern Prov. Njuki-ini Forest, nr. Forest station, 1455m 0.51660o S, 37.41843o E 15—29.IX.2008 MT R. Copeland
YMZ376_Leiophron_THA	Thailand, Trang Pr. Khoa Chang, Forest Research Stn. 7°33'2"N 99°47'23"E 75m 21—26.I.2005 D. Lohman
YMZ377_Leiophron_THA	Thailand, Trang Pr. Khoa Chang, Forest Research Stn. 7°33'2"N 99°47'23"E 75m VIII.2005 D. Lohman
YMZ378_Leiophron_THA	Thailand, Trang Pr. Khoa Chang, Forest Research Stn. 7°33'2"N 99°47'23"E 75m VII.2005 D. Lohman
YMZ380_Leiophron_CON	Congo, Dept Pool Iboubikro, Lesio-Looun Pk, 330m 03°16.196S, 015°28.267E MT 26.XI—7.XII.2008 Sharkey+Braet A131
YMZ382_Leiophron_KOR	South Korea, Ganwondo Chuncheon, Man-myeon Balsan, 300m, MT in forest 37°43.29'N 127°37.73'E 30.IX—11.XI.2006 Tripotin rec.
YMZ383_Leiophron_KOR	South Korea, Chungnam Daejon-si Wadong 36°24.02'N 127°25.98E 19.VI.—16.VII.2006 P. Tripotin, MT, Forest edge, wild rose patch
YMZ384_Leiophron_KOR	South Korea, Ganwondo Pyeonchang, Yongpyeong - myeon Nodong Valley, 900m 37°42.08'N 128°28.89'E 31.V.—5.VI.2006 P. Tripotin, MT in forest
YMZ385_Leiophron_KOR	South Korea, Ganwondo Pyeonchang, Yongpyeong - myeon Nodong Valley, 900m 37°42.08'N 128°28.89'E 31.V.—5.VI.2006 P. Tripotin, MT in forest
YMZ386_Leiophron_CON	Congo, Dept Pool Iboubikro, Lesio-Looun Pk, 330m 03°16.196S, 015°28.267E MT4 20.X.2008 Sharkey+Braet A134

Taxon Label	Locality Label (verbatim)
YMZ388_Leiophron_pallidistigma_KOR	South Korea, Chungnam Daejon-si Wadong 36°24.02'N 127°25.98E 28.V.—19.VI.2006 P. Tripotin, MT, Forest edge, wild rose patch
AB102 Microctonus (Perilitini)	USA, Kentucky, Lexington, Tee It Up Golf 37°58'39"N 84°24'59"W MT [HI#200] 10—17.IX.2004, B. Sharanowski
JS01000218 Townesilitus (Townesilitini)	Sweden, Sk, Ystads kommun, Sandhammaren strand, Järahusen.Border between forest and sandhill dunes. N61°42.074´ E13°98.890´ (= TrapID 1005) 22.v - 15.vii.2005 (=coll. event ID 1419)
JS115 Chrysopophthorus (Helorimorphini)	Colombia, Magdelena, PNN Tayrona Canaveral (30m). 3-22.xi.2000

APPENDIX B: CHAPTER THREE SUPPORTING INFORMATION

Supplementary Table 3.1. List of Nearctic species of the *Peristenus pallipes* complex, their distribution, host, and peak flight time according to Goulet and Mason (2006).

Species	Species group	Distribution	Flight Period	Voltinism	Host(s)	Provincial/State Record
P. dayi	*dayi*	Eastern NA	Early May - Mid Jun	Uni, 1st generation	*Adelphocoris lineolatus*, *Lygus lineolaris*	ON, QC, DE, NJ, NY
P. braunae	*dayi*	Northern NA	Early Jun - Mid Aug	Uni, 1st generation	*A. lineolatus*, *L. lineolaris*	AB, BC, MB, NB, NS, NT, QC, SK, AK, CA, CO, UT
P. mellipes	*mellipes*	Eastern NA	Early May - Early Jul	Uni, 1st generation	*A. lineolatus*, *L. lineolaris*	NB, NS, QC, ON, DE, GA, IL, KS, MA, ME, MI, MO, MS, NC, NJ, NY, VA
P. pseudopallipes	*mellipes*	Eastern NA	Mid Jul - Mid Sep	Uni, 2nd generation	*L. lineolaris*, *L. vanduzeei*	NB, QC, ON, CT, DE, GA, NC, NJ, NY, OH
P. otaniae	*mellipes*	Northern NA	Early Jun - Early Aug	Uni, 1st generation	*A. lineolatus*, *L. lineolaris*, *Lygus* spp.	AB, BC, MB, NF, QC, SK, CO
P. broadbenti	*mellipes*	Western NA	Late Jun - Late Aug	Uni, 2nd generation	*Lygus* spp.	AB, BC, MT, NV, OR, WA, WY
P. carcamoi	*mellipes*	Western NA	Late May - Late Jun	Uni, 1st generation	*Lygus* spp.	AB, BC
P. gillespiei	*mellipes*	Western NA	Early May - Late Jun	Uni	*Lygus* spp.	BC, CA
P. howardi	*mellipes*	Western NA	Early May - Late Sepr	Bi	*L. hesperus*	ID, WA

Supplementary Table 3.2. Specimens of *Peristenus* used in this study, including the collection information and the gene that were amplified. Type specimens indicated using #, specimens from the Barcode of Life Database (BOLD) is indicated using *.

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
10BBCHY-2928*	*P. braunae*	*P. dayi*	JN293532		CAN: Saskatchewan 7/16/2010
10BBCHY-2929*	*P. braunae*	*P. dayi*	KR801414		CAN: Saskatchewan 7/16/2010
10BBCHY-2930*	*P. braunae*	*P. dayi*	JN293533		CAN: Saskatchewan 7/16/2010
BBHYE981-10*	*P. pseudopallipes*	*P. mellipes*	HQ552500		CAN: Nova Scotia 7/27/2009
BBHYF343-10*	*P. pseudopallipes*	*P. mellipes*	HQ929460		CAN: New Brunswick 8/6/2009
BIOUG01018-G02*	*P. dayi*	*P. dayi*	KR806328		CAN: Ontario 5/5/2010
BIOUG01638-H09*	*P. mellipes*	*P. mellipes*	KR789606		CAN: Ontario 6/20/2011
BIOUG01638-H10*	*P. mellipes*	*P. mellipes*	KR797737		CAN: Ontario 6/20/2011
BIOUG01688-A08*	*P. mellipes*	*P. mellipes*	KR794605		CAN: Ontario 6/20/2011
BIOUG01688-B01*	*P. mellipes*	*P. mellipes*			CAN: Ontario 6/20/2011
BIOUG01688-C02*	*P. mellipes*	*P. mellipes*			CAN: Ontario 6/20/2011
BIOUG01688-C07*	*P. mellipes*	*P. mellipes*	KR808800		CAN: Ontario 6/20/2011
BIOUG05726-D09*	*P. dayi*	*P. dayi*	KR884749		CAN: Ontario 5/13/2011
BIOUG08726-A04*	*P. pseudopallipes*	*P. mellipes*			CAN: Ontario: 7/30/2004
BIOUG08726-A07*#	*P. gillespiei*	*P. howardi*			USA: Idaho 6/18/2000
BIOUG08726-A10*	*P. braunae*	*P. dayi*			CAN: Alberta 5/12/2005
BIOUG08726-A11*#	*P. braunae*	*P. dayi*			CAN: Québec 5/16/2002
BIOUG08726-B01*#	*P. dayi*	*P. dayi*			CAN: Ontario 5/24/2003
BIOUG08726-B08*	*P. otaniae*	*P. mellipes*			CAN: Québec 6/24/2003
BIOUG08726-C05*	*P. howardi*	*P. howardi*			USA: Delaware 7/03/2003
CBRA0352*	*P. carcamoi*	*P. mellipes*	HM396916		CAN: Alberta 5/27/2005

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
CBRA0354*	*P. carcamoi*	*P. mellipes*	HM396917	KY566134	CAN: Alberta 6/2/2005
CBRA0360*	*P. otaniae*	*P. mellipes*	HM396918		CAN: Yukon Territories 7/5/2006
CBRA0361*	*P. otaniae*	*P. mellipes*	HM396919	KY566135	CAN: Yukon Territories 7/5/2006
CBRA0362*	*P. otaniae*	*P. mellipes*	HM396920	KY566136	CAN: Yukon Territories 7/6/2006
CBRA0364*	*P. otaniae*	*P. mellipes*		KY566137	CAN: Yukon Territories 7/6/2006
CBRA0366*	*P. otaniae*	*P. mellipes*	HM396921	KY566138	CAN: Yukon Territories 7/16/2006
CBRA0373*	*P. carcamoi*	*P. mellipes*	HM904913		CAN: Yukon Territories 7/3/2006
CBRA0386*	*P. pseudopallipes*	*P. mellipes*	HM396924		CAN: Newfoundland 7/13/2008
CBRA0387*	*P. pseudopallipes*	*P. mellipes*	HM396925		CAN: Newfoundland 7/13/2008
CBRA0388*	*P. pseudopallipes*	*P. mellipes*	HM396926		CAN: Newfoundland 7/16/2008
CBRA0390*	*P. pseudopallipes*	*P. mellipes*	HM396927		CAN: Newfoundland 7/18/2008
CBRA0392*	*P. pseudopallipes*	*P. mellipes*	HM396928		CAN: Newfoundland 7/16/2008
CBRA0394*	*P. braunae*	*P. dayi*	HM396930		CAN: Newfoundland 7/14/2008
CBRA0395*	*P. pseudopallipes*	*P. mellipes*	HM396931		CAN: Newfoundland 7/16/2008
CBRA0396*	*P. pseudopallipes*	*P. mellipes*	HM396932		CAN: Newfoundland 7/13/2008
CBRA0397*	*P. pseudopallipes*	*P. mellipes*	HM396933		CAN: Newfoundland 7/13/2008
CBRA0400*	*P. braunae*	*P. dayi*	HM396936		CAN: Newfoundland 7/17/2008
CBRA0401*	*P. braunae*	*P. dayi*	HM396937	KY566139	CAN: Newfoundland 7/18/2008
CBRA0402*	*P. braunae*	*P. dayi*	HM396938		CAN: Newfoundland 7/13/2008

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
CBRA0404*	*P. braunae*	*P. dayi*	HM396939		CAN: Newfoundland 7/12/2008
CBRA0405*	*P. braunae*	*P. dayi*	HM396940		CAN: Newfoundland 7/10/2008
CBRA0406*	*P. braunae*	*P. dayi*	HM396941		CAN: Newfoundland 7/11/2008
CBRA0411*	*P. pseudopallipes*	*P. mellipes*	HM396943		CAN: Prince Edward Island 9/12/2006
CBRA0412*	*P. dayi*	*P. dayi*	HM396944		CAN: Ontario 5/29/2008
CBRA0417*	*P. mellipes*	*P. mellipes*			CAN: Ontario 6/8/2007
CBRA0421*	*P. dayi*	*P. dayi*	HM396950		CAN: Ontario 6/3/2007
CBRA0422*	*P. dayi*	*P. dayi*	HM396951		CAN: Ontario 6/3/2007
CBRA0423*	*P. dayi*	*P. dayi*	HM396952		CAN: Ontario 6/3/2007
CBRA0424*	*P. dayi*	*P. dayi*	HM396953		CAN: Ontario 6/4/2007
CBRA0425*	*P. dayi*	*P. dayi*	HM396954		CAN: Ontario 6/3/2007
CBRA0426*	*P. dayi*	*P. dayi*	HM396955		CAN: Ontario 6/3/2007
CBRA0427*	*P. dayi*	*P. dayi*	HM396956		CAN: Ontario 6/3/2007
CBRA0428*	*P. dayi*	*P. dayi*	HM396957		CAN: Ontario 6/3/2007
CBRA0429*	*P. dayi*	*P. dayi*	HM396958		CAN: Ontario 6/3/2007
CBRA0430*	*P. dayi*	*P. dayi*	HM396959		CAN: Ontario 6/3/2007
CBRA0431*	*P. dayi*	*P. dayi*	HM396960		CAN: Ontario 6/3/2007
CBRA0432*	*P. dayi*	*P. dayi*	HM396961		CAN: Ontario 6/3/2007
CBRA0433*	*P. dayi*	*P. dayi*	HM396962		CAN: Ontario 6/3/2007
CBRA0434*	*P. dayi*	*P. dayi*	HM396963		CAN: Ontario 6/3/2007
CBRA0435*	*P. dayi*	*P. dayi*	HM396964		CAN: Ontario 6/3/2007
CBRA0440*	*P. mellipes*	*P. mellipes*	HQ941771		CAN: Ontario 6/16/2007
CBRA0442*	*P. mellipes*	*P. mellipes*	HM396951		CAN: Ontario 6/16/2007
CBRA0443*	*P. mellipes*	*P. mellipes*	HQ941773		CAN: Ontario 6/16/2007
SAHYM202-10*	*P. braunae*	*P. dayi*	HQ972328		CAN: Manitoba 7/21/2009

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
SAHYM252-10*	*P. otaniae*	*P. mellipes*	HQ972354		CAN: Manitoba 7/31/2009
SAHYM277-10*	*P. braunae*	*P. dayi*	HQ972372		CAN: Manitoba 7/20/2009
SAHYM380-10*	*P. braunae*	*P. dayi*	HQ972448		CAN: Manitoba 7/31/2009
SSWLA2620_13*	*P. broadbenti*	*P. howardi*	KR891177		CAN: Alberta 6/27/2012
SSWLA3665_13*	*P. broadbenti*	*P. howardi*	KR901182		CAN: Alberta 6/27/2012
YMZ039	*P. relictus*	*P. relictus*	KY566081	KY566140	GER: Schleswig-Holstein 8/11/2001
YMZ080	*Euphoriella sp.*	*Euphoriella sp.*	KY566080	KY566133	CAN: Manitoba 6/17/2013
YMZ085	*Leiophron sp.*	*Leiophron sp.*	KY566082	KY566141	CAN: Manitoba 7/5/2012
YMZ086	*P. otaniae*	*P. mellipes*	KY566083	KY566142	CAN: Manitoba 8/1/2013
YMZ087	*P. carcamoi*	*P. mellipes*	KY566084	KY566143	CAN: Alberta 7/6/2012
YMZ088	*P. carcamoi*	*P. mellipes*	KY566085	KY566144	CAN: Alberta 7/6/2012
YMZ089	*P. carcamoi*	*P. mellipes*	KY566086	KY566145	CAN: Alberta 7/6/2012
YMZ090	*P. carcamoi*	*P. mellipes*	KY566087	KY566146	CAN: Alberta 7/6/2012
YMZ122	*P. otaniae*	*P. mellipes*	KY566088	KY566147	CAN: Manitoba 7/10/2014
YMZ123	*P. otaniae*	*P. mellipes*	KY566089	KY566148	CAN: Manitoba 7/10/2014

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
YMZ124	*P. otaniae*	*P. mellipes*	KY566090	KY566149	CAN: Manitoba 7/10/2014
YMZ125	*P. otaniae*	*P. mellipes*	KY566091		CAN: Manitoba 7/10/2014
YMZ127	*P. otaniae*	*P. mellipes*	KY566092		CAN: Manitoba 8/15/2014
YMZ159	*P. braunae*	*P. dayi*	KY566093	KY566150	CAN: Manitoba 6/28/2013
YMZ161	*P. braunae*	*P. dayi*	KY566094	KY566151	CAN: Manitoba 6/28/2013
YMZ164	*P. braunae*	*P. dayi*	KY566095	KY566152	CAN: Manitoba 6/28/2013
YMZ165	*P. braunae*	*P. dayi*	KY566096	KY566153	CAN: Manitoba 6/28/2013
YMZ206	*P. braunae*	*P. dayi*	KY566097		CAN: Manitoba 6/25/14
YMZ211	*P. braunae*	*P. dayi*	KY566098	KY566154	CAN: Manitoba 7/7/2012
YMZ223	*P. braunae*	*P. dayi*	KY566109		CAN: Manitoba 6/30/2015
YMZ224	*P. otaniae*	*P. mellipes*	KY566115		CAN: Manitoba 6/30/2015
YMZ225	*P. otaniae*	*P. mellipes*	KY566116		CAN: Manitoba 6/30/2015
YMZ226	*P. otaniae*	*P. mellipes*	KY566111		CAN: Manitoba 6/30/2015
YMZ227	*P. otaniae*	*P. mellipes*	KY566117		CAN: Manitoba 6/30/2015
YMZ228	*P. otaniae*	*P. mellipes*	KY566113		CAN: Manitoba 6/30/2015
YMZ230	*P. otaniae*	*P. mellipes*	KY566114		CAN: Manitoba 6/30/2015

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
YMZ231	*P. otaniae*	*P. mellipes*	KY566110		CAN: Manitoba 6/30/2015
YMZ232	*P. otaniae*	*P. mellipes*	KY566112		CAN: Manitoba 6/30/2015
YMZ243	*P. carcamoi*	*P. mellipes*	KY566118		CAN: Alberta 6/30/2015
YMZ244	*P. carcamoi*	*P. mellipes*	KY566127		CAN: Alberta 6/30/2015
YMZ245	*P. carcamoi*	*P. mellipes*	KY566126		CAN: Alberta 6/30/2015
YMZ246	*P. carcamoi*	*P. mellipes*	KY566119		CAN: Alberta 6/30/2015
YMZ249	*P. carcamoi*	*P. mellipes*	KY566124		CAN: Alberta 6/30/2015
YMZ250	*P. carcamoi*	*P. mellipes*	KY566120		CAN: Alberta 6/30/2015
YMZ251	*P. carcamoi*	*P. mellipes*	KY566121		CAN: Alberta 6/30/2015
YMZ252	*P. carcamoi*	*P. mellipes*	KY566125		CAN: Alberta 6/30/2015
YMZ263	*P. broadbenti*	*P. howardi*	KY566132		CAN: Alberta 8/8/2015
YMZ264	*P. broadbenti*	*P. howardi*	KY566128		CAN: Alberta 8/8/2015
YMZ265	*P. broadbenti*	*P. howardi*	KY566131		CAN: Alberta 8/8/2015
YMZ267	*P. broadbenti*	*P. howardi*	KY566130		CAN: Alberta 8/8/2015
YMZ269	*P. broadbenti*	*P. howardi*	KY566129		CAN: Alberta 8/8/2015
YMZ270	*P. broadbenti*	*P. howardi*	KY566122		CAN: Alberta 8/8/2015
YMZ271	*P. broadbenti*	*P. howardi*	KY566123		CAN: Alberta 8/8/2015
YMZ333	*P. carcamoi*	*P. mellipes*	KY566099	KY566155	CAN: Alberta 7/14/2011
YMZ335	*P. broadbenti*	*P. howardi*	KY566100	KY566156	CAN: Alberta 7/14/2011
YMZ336	*P. broadbenti*	*P. howardi*	KY566101	KY566157	CAN: Alberta 8/10/2011
YMZ337	*P. broadbenti*	*P. howardi*	KY566102	KY566158	CAN: Alberta 8/10/2011

Specimen #	Original Species Name	Revised Species Name	COI	CytB	Collection Info
YMZ338	*P. broadbenti*	*P. howardi*	KY566103	KY566159	CAN: Alberta 8/10/2011
YMZ339	*P. broadbenti*	*P. howardi*	KY566104		CAN: Alberta 8/10/2011
YMZ340	*P. broadbenti*	*P. howardi*	KY566105	KY566160	CAN: Alberta 8/10/2011
YMZ341	*P. relictus*	*P. relictus*	KY566106	KY566161	USA: New Jersey 6/9/2016 (Lab colony)
YMZ354	*Leiophron sp.*	*Leiophron sp.*	KY566107	KY566162	USA: Kentucky 9/29/2010
YMZ355	*P. dayi*	*P. dayi*	KY566108	KY566163	USA: Kentucky 5/22/2008

Supplementary Table 3.3. A; Intraspecific divergence of *COI* and *CytB* for the *Peristenus* species calculated using the Kimura-2-Parameter (K2P). B; Interspecific divergence of *COI* (1st value) and *CytB* (2nd value) for the *Peristenus* species calculated using the Kimura-2-Parameter (K2P).

A.

Groups	**COI (%)**	**CytB (%)**
P. dayi	1.7	0.8
P. mellipes	1.6	0.3
P. howardi	0.9	0.2
Outgroups	16.6	20.2

B.

Groups	***P. dayi* (%)**	***P. mellipes* (%)**	***P. howardi* (%)**
P. dayi			
P. mellipes	10.2/10.0		
P. howardi	9.7/8.2	4.2/5.3	
Outgroups	17.8/19.3	17.9/19.8	17.8/19.0

Supplementary Table 3.4. The best separating ratios for females of *Peristenus dayi* and *Peristenus mellipes* complex.

Ratios	***P. dayi***				***P. mellipes* group**			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
eye.d/eye.b	1.14	1.38	1.25	0.05	0.9	1.24	1.05	0.08
eye.h/gsp.l	1.31	1.5	1.4	0.06	1.45	1.86	1.64	0.11

APPENDIX C: CHAPTER FOUR SUPPORTING INFORMATION

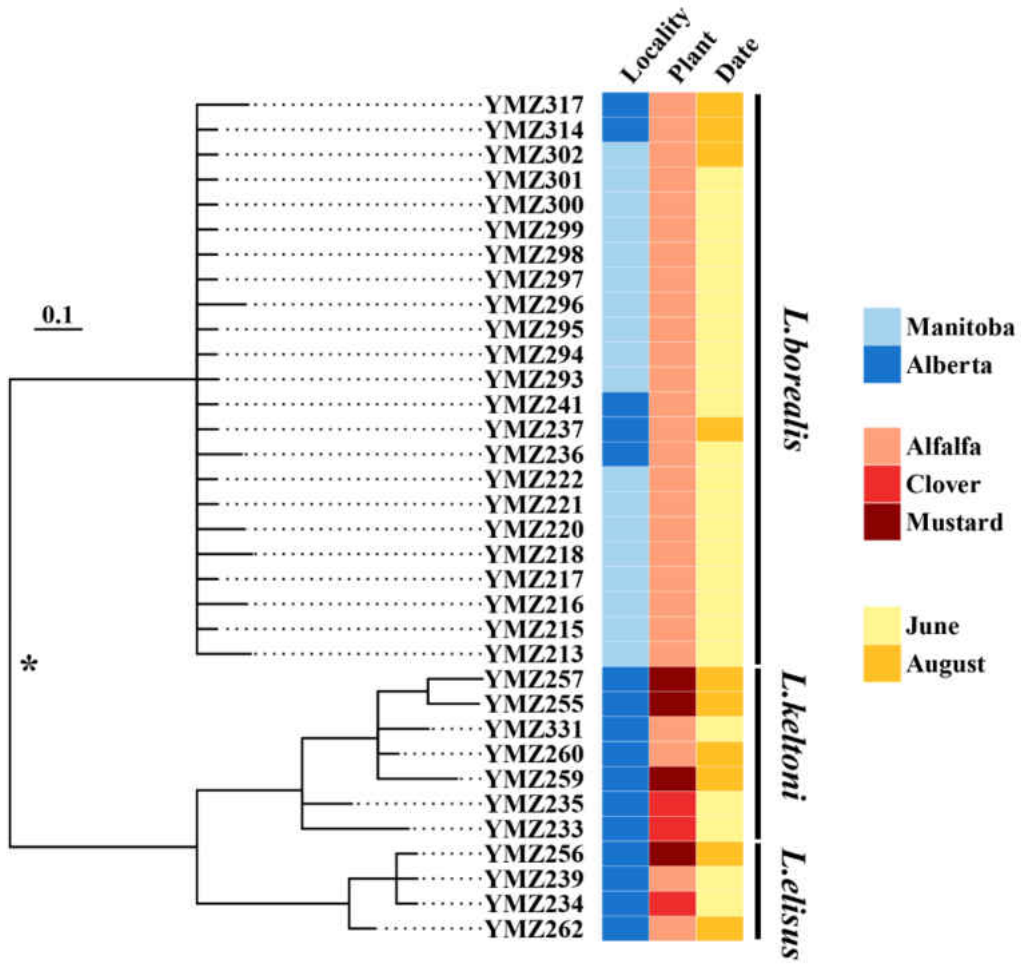


Figure A4.1. Inferred phylogeny of *Lygus* species from the MrBayes analysis of the *COI* data. Asterisk indicates posterior probability of value of ≥ 0.98. Sampling locality is colored coded in shades of blue, host plant in shades of red, and collecting date is in shades of yellow.

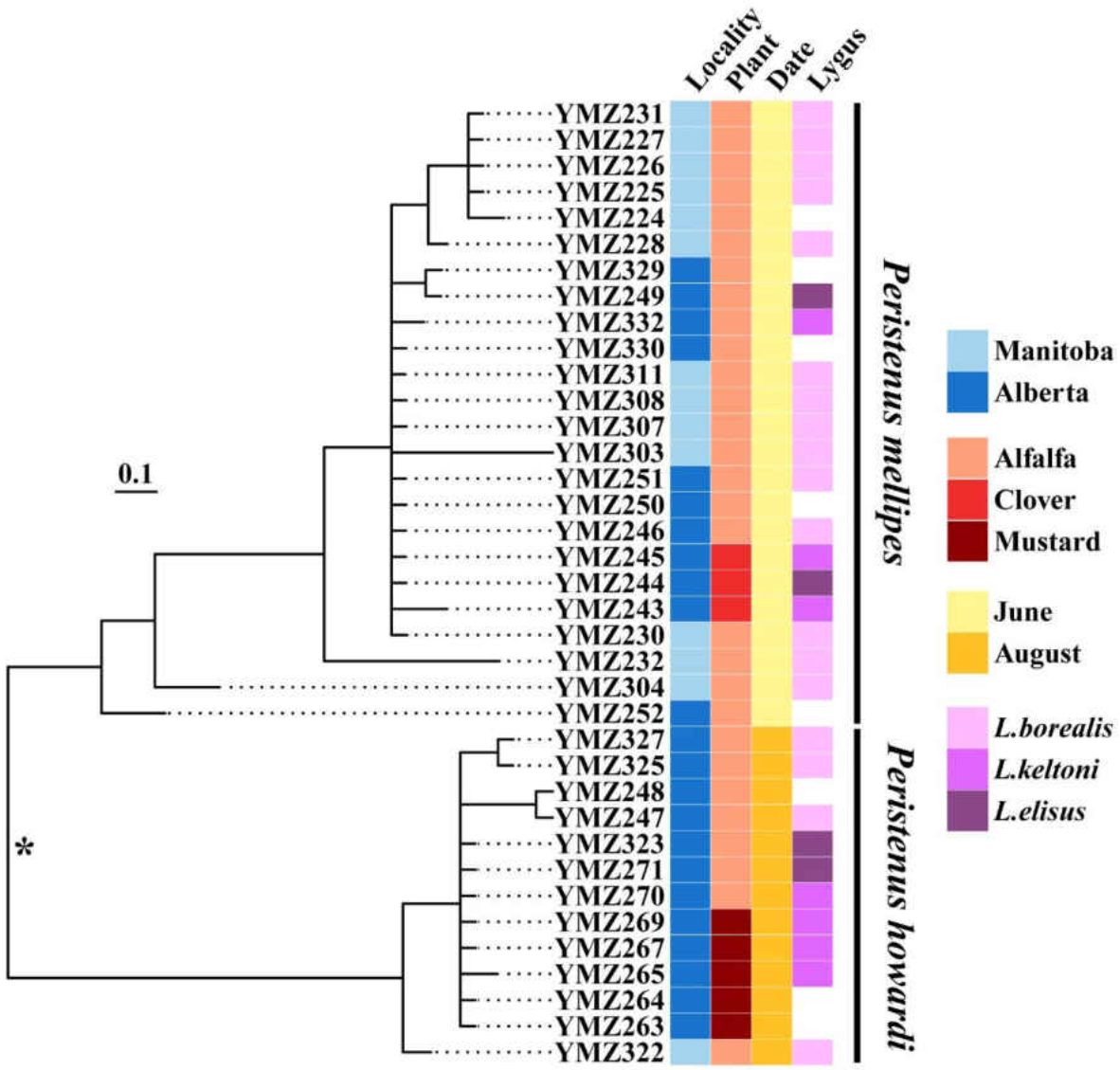


Figure A4.2. Inferred phylogeny of *Peristenus* species from the MrBayes analysis of the *COI* data. Asterisk indicates posterior probability of value of ≥ 0.98. Sampling locality is colored coded in shades of blue, host plant in shades of red, collecting date is in shades of yellow, and host bug is in shades of pink.