Cryptic species status of *Anopheles* (Diptera: Culicidae) mosquitoes
in Canada using a multidisciplinary approach

Aynsley Thielman, BSc

A thesis
submitted to the Department of Biological Sciences
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Faculty of Mathematics and Science, Brock University
St. Catharines, Ontario

© 2011

Abstract

Many species of *Anopheles* mosquitoes (Diptera: Culicidae) are now recognized as species complexes whose members are often indistinguishable morphologically but identifiable based on ecological, genetic, or behavioural data. Because the members of species complexes often differ in their vector potential, accurate identification of vector species is essential for successful mosquito control. To investigate the cryptic species status of *Anopheles* mosquitoes in Canada, specimens were collected from across the country and examined using morphological, molecular, and ecological data.

Six of the seven traditionally recognised species from Canada were collected from locations in British Columbia, Quebec, Newfoundland and Labrador, and throughout Ontario, including *Anopheles barberi, An. earlei, An. freeborni, An. punctipennis, An. quadrimaculatus s.l.*, and *An. walkeri.* Variation in polymorphic traits within *An. earlei, An. punctipennis,* and *An. quadrimaculatus s.l.* were quantified and egg morphology examined using scanning electron microscopy. Morphological identification of adult and larval specimens suggested that two described cryptic species, *An. perplexens* and *An. smaragdinus*, were present in Canada.

DNA sequence data were analysed for evidence of cryptic species using three molecular markers: COI, ITS2, and ITS1. Intraspecific COI variation was very low in most species (<1%), except for *An. punctipennis* with 2% sequence divergence between those from British Columbia (BC) and Ontario (ON), and *An. walkeri* with 7% sequence divergence between populations from Manitoulin Island (NO) and Long Point Provincial Park (LP). Similar patterns were also seen using ITS2 and ITS1. Therefore, molecular

data revealed the presence of two putative cryptic species within two species examined (i.e., *An. walkeri* and *An. punctipennis*), corresponding to collection location (i.e., NO vs. LP and BC vs. ON, respectively). Surprisingly, there was no molecular support for the presence of either *An. perplexens* or *An. smaragdinus* in Canada despite the morphological assessments.

Ecological data from all collection sites were recorded and are available in an online database designed to manage all collection and identification data. Current bionomic information, including regional abundance, larval habitat, and species associations, was determined for each species. This multidisciplinary study of *Anopheles* mosquitoes is the first detailed investigation of these potential disease vectors in Canada and demonstrates the importance of an integrated approach to anopheline systematics that includes molecular data.

Preface

This PhD thesis was prepared in monograph format. I planned and completed all aspects of this research, with the help of many field and research assistants (see Acknowledgements), for which Dr. Hunter provided mentorship and financial support. This thesis is organized into five chapters and includes an introductory chapter, three data chapters, and a concluding chapter, with manuscripts for each data chapter in preparation for submission to academic journals. Specimens and collection data were deposited in the Canadian National Collection of Insects, Agriculture and Agri-Food Canada in Ottawa, Ontario, Canada.

Chapter One provides an introduction to systematic investigations of *Anopheles* (Diptera: Culicidae) mosquitoes and the significance of cryptic species within this medically important group of insects. It includes a general overview of mosquitoes and the systematics of these potential disease vectors, followed by a discussion of cryptic species, the methods used to distinguish isomorphic members of cryptic species complexes, and the importance of ecological data in such investigations. Also included is a discussion regarding the use of an integrated approach to *Anopheles* systematics, descriptions of all species known from or suspected to occur in Canada, and the objective of this study.

Chapter Two is the first of three data chapters and involves the morphological analysis of *Anopheles* mosquitoes collected from across Canada. It provides background information on egg, larval, and adult morphology, and on potential cryptic species in Canada, and describes the methods used to collect and identify specimens for analysis. The results of morphological analyses for each species are discussed, followed by a summary and conclusions with respect to morphological data.

Chapter Three is the second data chapter and involves the examination of molecular data for evidence of cryptic species within the *Anopheles* species present in Canada. It provides an introduction to molecular systematics of anophelines and the molecular markers used in this study, i.e., an 800bp portion of the mitochondrial cytochrome *c* oxidase I gene (COI) and the ribosomal internal transcribed spacer sequences 1 and 2 (ITS1 and ITS2), followed by a review of molecular studies involving

Anopheles species present in Canada. The materials and methods used to analyse these molecular markers are described, including the primers and PCR conditions, how consensus sequences were determined, and analysis of the resulting nucleotide sequences. The results for each molecular marker examined are discussed, followed by a summary and the conclusions based on molecular data.

Chapter Four is the third and final data chapter of this thesis and involves the analysis of ecological data associated with the larval habitats of *Anopheles* species in Canada. While these data were not analysed for evidence of cryptic species *per se*, they represent the first detailed analysis of the larval habitats of the *Anopheles* species present in Canada in the northern parts of these species North American ranges. Ecological data associated with larval and adult mosquito collections were recorded and an online database was designed to maintain this data. Chapter Four focuses on the analysis larval habitat data, including water body type, emergent and floating vegetation type, and species associations for each species examined in the study. The results for each larval habitat characteristic examined are discussed, followed by a summary of the analyses of ecological data.

Chapter Five is the final chapter of this thesis and involves a comparison of the results of the different methods used to examine the cryptic species status of anophelines in Canada, i.e., morphological, molecular, and ecological. It includes a review of the results of each of these methods, highlighting similarities and incongruence among them. The importance of an integrated approach to *Anopheles* systematics is discussed, and the overall conclusions of the study provided.

Acknowledgements

First and foremost, I would like to thank Dr. Fiona Hunter for introducing me to fascinating world of insects, especially mosquitoes, and for all her support over the years. It was her constant encouragement, and her undying confidence in me (and my work), that inspired me to "keep on truckin'" during the harder times.

I would also like to thank the members of my thesis advisory committee, including present members, Dr. John Middleton and Dr. Dan McCarthy, and past members Dr. Jean Richardson and Dr. Miriam Richards. Their comments and suggestions regarding my thesis during the course of my graduate studies at Brock University are greatly appreciated.

My deep gratitude also goes to Alina Cywinska, whose help both in the field and with molecular techniques is greatly appreciated. Her passion and determination definitely rubbed off on me during the years we worked together, and helped me to persevere when things got rough. Her level-headedness about science, and life in general, will never be forgotten.

Thanks also go to the many undergraduate field and research assistants that I've had the pleasure of working with over the years, including Christina Mychaskiw and Owen Hughes, as well as other field and research assistants, including my friends Wayne Rail, Lauren Pinault, Greg Gunn, Jared Spagnolo, and Layla Thielman. Wayne, Greg, Christina, Lauren, Jared, Layla, and Alina assisted with the collection of specimens in the field, and Christina, Owen, and Greg helped with maintaining live specimens in the laboratory, and preserving and identifying the specimens collected. I would also like to thank my lab mates, Daniel Antwi-Amoabeng, Benyam Abraham, Donny Aedy, and Roger Kroeker, for their comments and feedback on my research, as well as Lauren Pinault for her constructive criticism, academic advice, and technical assistance with mapping programs.

My appreciation extends to other members of the Brock University community and beyond, including Glenda Hooper, the Scanning Electron Microscope technician who helped obtain the SEM images used in this thesis, and Ming Lin, the Computer Science technician who designed the online database that maintains all data associated with my

mosquito collections. I am also grateful to Drs. Doug Currie, Chris Darling, and Antonia Guidotti, and Julio Rivera, at the Royal Ontario Museum for their help with the Entovision automontage microscope-camera system that was used to obtain the mosquito images included in this thesis.

For funding this research, I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for a Discovery Grant to Dr. Hunter and a Doctoral Scholarship to myself, and the Ontario Graduate Scholarship in Science and Technology (OGSST).

I would also like to thank all my friends and fellow graduate students for their friendship and academic assistance throughout my time here at Brock University, including Lauren Pinault, Amy Sharp, Curtis Russell, Sandra Rehan, Jessica Vickruck, Chris Course, Viviana Cadena, and Amanda Lepp. Your lunchtime antics and social retreats provided much-needed comic relief from the stress that being a graduate student can bring.

Last, but certainly not least, is my family. I am extremely grateful for my partner, Jared Spagnolo, and daughter, Layla, whose support, encouragement, patience and understanding made sure that my success was possible. I would like to thank my father, Al Thielman, and his partner, Marnie Newport, for their support and encouragement over the years as well. This thesis is dedicated to my mother, Jacqueline, who instilled in me the belief that I could achieve anything that I wanted in life.

Table of Contents

Abstract ii

Preface iv

Acknowledgements vi

Table of Contents viii

List of Tables xii

List of Figures xiv

Chapter 1: Introduction to *Anopheles* systematics 1

1.1. Introduction to mosquitoes (Diptera: Culicidae) 1

1.2. Mosquito systematics 5

1.3. Species complexes and cryptic species 11

1.4. Ecological associations 13

1.5. Integrated approach to *Anopheles* systematics 15

1.6. *Anopheles* mosquitoes known from Canada 16

1.6.1. *Anopheles barberi* Coquillett, 1903 17

1.6.2. *Anopheles crucians s.l.* Wiedermann, 1828 18

1.6.3. *Anopheles earlei* Vargas, 1943 18

1.6.4. *Anopheles freeborni* Aitken, 1939 20

1.6.5. *Anopheles punctipennis* (Say, 1823) 20

1.6.6. *Anopheles quadrimaculatus s.s.* Say, 1824 22

1.6.7. *Anopheles walkeri* Theobold, 1901 24

1.7. *Anopheles* species suspected to occur in Canada 24
1.7.1. *Anopheles occidentalis* Dyar and Knab 1906 25
1.7.2. *Anopheles perplexens* Ludlow, 1907 25
1.7.3. *Anopheles smaragdinus* Reinert 1999 27
1.8. Status of knowledge in Canada 29
1.9. Summary 30
Chapter 2: Morphological analyses of *Anopheles* mosquitoes from Canada 31
2.1. Introduction 31
2.1.1. Egg morphology 32
2.1.2. Larval morphology 34
2.1.3. Adult morphology 36
2.1.4. Cryptic Species Potentially Occurring in Canada 38
2.2. Methods 41
2.2.1. Mosquito collections 41
2.2.2. Species identification 46
2.2.3. Scanning electron microscopy of eggs 49
2.2.4. Isofemale progeny broods (IPBs) 51
2.3. Results and Discussion 51
2.3.1. *An. barberi* 52
2.3.2. *An. crucians s.l.* 52
2.3.3. *An. earlei* 54
2.3.4. *An. freeborni* 58
2.3.5. *An. punctipennis* 58

2.3.6. *An. quadrimaculatus s.l.* 65

2.3.7. *An. walkeri* 67

2.3.8. SEM Egg data 69

2.4. Conclusions and Summary 72

Chapter 3: Comparison of ribosomal and mitochondrial DNA in *Anopheles* (Diptera: Culicidae) mosquitoes from Canada 77

3.1. Introduction to molecular systematics 77

3.1.1. Mitochondrial COI 81

3.1.2. Ribosomal ITS2 and ITS1 83

3.1.3. Canadian anophelines 86

3.2. Materials and Methods 87

3.2.1. Mosquito collections and identification 87

3.2.2. Specimen preparation and DNA extraction 87

3.2.3. Primers and PCR conditions 88

3.2.4. PCR products and sequencing 92

3.2.5. Consensus sequence determination 92

3.2.6. Data analyses 93

3.3. Results 94

3.3.1. COI 94

3.3.2. ITS2 105

3.3.3. ITS1 111

3.4. Discussion 119

3.5. Summary and conclusions 128

Chapter Four: Ecological analyses of the larval habitats of *Anopheles* species in Canada **130**

4.1. Introduction 130

4.2. Materials and Methods 134

4.3. Results and Discussion 139

4.3.1. Overall species abundance 139

4.3.2. Regional species abundance 143

4.3.3. Larval habitat analyses 145

4.3.4. Emergent and floating vegetation 157

4.3.5. Species associations 161

4.4 Summary 164

Chapter 5: Morphological, molecular, and ecological data: Results of an integrated approach to *Anopheles* systematics in Canada **168**

5.1. Introduction 168

5.2. Morphology 171

5.3. Molecular data 174

5.4. Ecology 177

5.5. Importance of an integrated approach 180

5.6. Conclusions 184

Bibliography **186**

Appendix I. Collection location/region codes **199**

Appendix II. Online Database **200**

Appendix III. Specimens used in molecular analyses **202**

LIST OF TABLES

Chapter Two

Table 2.1. Character matrix for the identification of *Anopheles* larvae 48

Table 2.2. Number of additional accessory tergal plates in *An. earlei* larvae 57

Table 2.3. Relative proportions of *An. punctipennis* and *An. perplexens* adults reared from larval dipping collections 63

Table 2.4. Relative proportion of larval and adult *An. perplexens* and *An. punctipennis* reared from isofemale progeny broods 63

Table 2.5. Identifications of *An. punctipennis* and *An. perplexens* females that produced IPBs, including SCP length and egg identifications 64

Table 2.6. Number and relative proportion of *An. quadrimaculatus s.l.* larvae identified using the larval key of Darsie and Ward (2005) 68

Table 2.7. Number and relative proportion of *An. quadrimaculatus s.l.* adults identified using the adult key of Darsie and Ward (2005) 70

Table 2.8. Identifications of *An. quadrimaculatus s.l.* females that produced IPBs, including number of tubercles and egg identifications 73

Chapter Three

Table 3.1. Volumes and concentrations of solutions used in preparation of PCR reactions for amplification of COI, ITS2 and ITS1 sequences 89

Table 3.2. List of primers used to amplify and sequence COI, ITS2, and ITS1 91

Table 3.3. Number of specimens for each species from each collection region for which COI sequences were obtained 95

Table 3.4. Average base composition (over all codons) of COI by each species 95

Table 3.5. Kimura two-parameter (K2P) sequence divergence levels in COI sequences for each species examined 97

Table 3.6. Kimura two-parameter (K2P) sequence divergence levels between each *Anopheles* species examined, and one *Culex* species as an outgroup 104

Table 3.7. Number of each species for which ITS2 sequences were obtained 107

Table 3.8. Summary statistics of ITS2 sequences from all anophelines examined 109

Table 3.9. p-Distance sequence divergence levels and number of unique sequences and variable sites among ITS2 sequences for all species examined 109

Table 3.10. Size and intensity of *An. punctipennis* and *An. perplexens* ITS1 bands 118

Chapter Four

Table 4.1. Known larval habitats of anopheline species present in Canada 133

Table 4.2. Larval habitat water body type classifications used for all *Anopheles*-positive larval dipping collections 136

Table 4.3. Vegetation types recorded during 2005-2009 larval dipping collections 137

Table 4.4. Number of larvae and adults obtained from all 2005-2009 larval dipping collections for all species examined 139

Table 4.5. Number of specimens collected from the five main sampling regions 143

Table 4.6. Number of *Anopheles* specimens collected from each water body type 146

Table 4.7. Relative frequencies of species co-occurrences among the anopheline species collected in *Anopheles*-positive larval dipping collections 162

LIST OF FIGURES

Chapter One

Figure 1.1. Lateral view of an adult female mosquito, *Anopheles earlei* 3

Figure 1.2. Scanning electron micrograph of the ventral surface of a mosquito egg 3

Figure 1.3. Dorsal view of a fourth instar larva, *Anopheles earlei* 4

Figure 1.4. Lateral view of a mosquito pupa, *Anopheles punctipennis* 4

Figure 1.5. Phylogeny of Nearctic *Anopheles* based on D2 region of 28S rRNA 8

Figure 1.6. Proposed classification of *Anopheles* mosquitoes from Canada 9

Figure 1.7. Geographic distribution of *Anopheles barberi* in North America 19

Figure 1.8. Geographic distribution of *Anopheles crucians s.l.* in North America 19

Figure 1.9. Geographic distribution of *Anopheles earlei* in North America 21

Figure 1.10. Geographic distribution of *Anopheles freeborni* in North America 21

Figure 1.11. Geographic distribution of *Anopheles punctipennis* in North America 23

Figure 1.12. Geographic distribution of *Anopheles quadrimaculatus s.s.* in North America 23

Figure 1.13. Geographic distribution of *Anopheles walkeri* in North America 26

Figure 1.14. Geographic distribution of *Anopheles occidentalis* in North America 26

Figure 1.15. Geographic distribution of *Anopheles perplexens* in North America 28

Figure 1.16. Estimated distribution of *Anopheles smaragdinus* in North America 28

Chapter Two

Figure 2.1. Eggs of *Anopheles punctipennis* and *Anopheles perplexens* (ventral view) 33

Figure 2.2. Eggs of *Anopheles quadrimaculatus s.s.* and *Anopheles smaragdinus* (ventral view) 33

Figure 2.3. Respiratory siphon of *Anopheles* and *Aedes* larvae (lateral view) 35

Figure 2.4. Common setal types of *Anopheles* larvae (dorsal view) 35

Figure 2.5. Additional accessory tergal plates in *An. freeborni* and *An. earlei* larvae (dorsal view) 37

Figure 2.6. Morphology of *Anopheles* and *Aedes* adult females (lateral view) 37

Figure 2.7. Larval head setae 2-C of *An. earlei* and *An. occidentalis* (dorsal view) 40

Figure 2.8. Wing morphology of *An. perplexens* and *An. punctipennis* (dorsal view) 40

Figure 2.9. Larval abdominal setae 2-IV,V of *An. perplexens* and *An. punctipennis* (dorsal view) 42

Figure 2.10. Characters used in discrimination of *An. quadrimaculatus s.s.* and *An. smaragdinus* (dorsal view) 42

Figure 2.11. Four main *Anopheles* collection regions in Canada 43

Figure 2.12. Four main and four incidental *Anopheles* collection locations in Ontario .. 43

Figure 2.13. Adult trapping and larval dipping collection sites during 2005-2009 field seasons 44

Figure 2.14. Mosquito breeders used to rear *Anopheles* larvae and adults 47

Figure 2.15. CDC light traps used to collect adult females mosquitoes 47

Figure 2.16. Head setae 5-7C of *An. barberi* and *An. quadrimaculatus s.l.* larvae (dorsal view) 53

Figure 2.17. Wings of adult *An. barberi* and *An. walkeri* (dorsal view) 53

Figure 2.18. Distinguishing characters of *An. crucians s.l.* larvae and adults 55

Figure 2.19. Distinguishing characters of *An. earlei* larvae and adults 55

Figure 2.20. Additional ATPs present in *An. earlei* larvae (dorsal view) 57

Figure 2.21. Distinguishing characteristics of *An. freeborni* larvae 59

Figure 2.22. Distinguishing characteristics of *An. freeborni* adults 59

Figure 2.23. Larval head setae 2-C of *An. punctipennis* and *An. walkeri* (dorsal view)... 61

Figure 2.24. Larval thoracic setae 1-P of *An. punctipennis* and *An. walkeri* (dorsal view) 61

Figure 2.25. Wing pattern variability among *An. punctipennis* siblings 63

Figure 2.26. Clypeal setae 2-C in *An. quadrimaculatus s.l.* larvae (dorsal view) 66

Figure 2.27. Abdominal setae 1-II of *An. quadrimaculatus* and *An. walkeri* larvae (dorsal view).. 66

Figure 2.28. Distinguishing characteristics of *An. quadrimaculatus s.l.* adults 68

Figure 2.29. Palpi of *An. walkeri* and *An. quadrimaculatus s.l.* (lateral view) 70

Figure 2.30. Eggs of *An. punctipennis* and *An. perplexens* (ventral view) 73

Figure 2.31. Eggs of *An. quadrimaculatus s.s.* (ventral view) 74

Chapter Three

Figure 3.1. Schematic diagram of the mtDNA genes COI and COII 84

Figure 3.2. Schematic diagram of rDNA subunits and spacer sequences 84

Figure 3.3. Neighbour-joining analysis of K2P distances of *An. barberi* COI sequences 97

Figure 3.4. Neighbour-joining analysis of K2P distances of *An. earlei* COI sequences 98

Figure 3.5. Neighbour-joining analysis of K2P distances of *An. freeborni* COI sequences 99

Figure 3.6. Neighbour-joining analysis of K2P distances of COI sequences from *An. punctipennis* and *An. perplexens* specimens.. 101

Figure 3.7. Neighbour-joining analysis of K2P distances of COI sequences from *An. quadrimaculatus s.l.* and *An. smaragdinus* specimens 102

Figure 3.8. Neighbour-joining analysis of K2P distances of COI sequences from *An. walkeri* specimens 104

Figure 3.9. Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of COI sequences from *Anopheles* mosquitoes collected in Canada 106

Figure 3.10. ITS2 PCR products for five *Anopheles* species 107

Figure 3.11. Clustal alignment of *An. earlei* ITS2 sequences (318bp) 110

Figure 3.12. Clustal alignment of BC and ON *An. punctipennis* ITS2 sequences 110

Figure 3.13. Clustal alignment of edited NO and LP *An. walkeri* ITS2 sequences 112

Figure 3.14. Clustal2W neighbour-joining tree of all species examined based on ITS2 112

Figure 3.15. ITS1 PCR products of all six *Anopheles* species examined 114

Figure 3.16. ITS1F and ITS1R regions sequenced in *An. earlei* and *An. freeborni* 114

Figure 3.17. ITS1 PCR products from 21 *An. punctipennis* specimens 117

Figure 3.18. ITS1 PCR products BC and NI *An. punctipennis* specimens 117

Figure 3.19. Clustal alignment of *An. quadrimaculatus s.l.* ITS1 sequences 120

Figure 3.20. Clustal alignment of LP and NO *An. walkeri* ITS1 sequences 121

Chapter Four

Figure 4.1. Number of *Anopheles* specimens obtained through all 2005-2009 larval dipping collections 143

Figure 4.2. Number of *An. barberi* larvae collected from each larval habitat type 146

Figure 4.3. Number of *An. freeborni* larvae collected from each larval habitat type 148

Figure 4.4. Number of *An. earlei* larvae collected from each larval habitat type 148

Figure 4.5. Number of *An. punctipennis* larvae collected from each larval habitat type 149

Figure 4.6. Number of *An. perplexens* larvae collected from each larval habitat type 149

Figure 4.7. Figure 4.7. Larval habitats in the Niagara region with exposed limestone 151

Figure 4.8. Map of alvar communities present in southern Ontario............................ 152

Figure 4.9. Number of *An. quadrimaculatus s.l.* larvae collected from each larval habitat type 154

Figure 4.10. Number of *An. walkeri* larvae collected from each larval habitat type 154

Figure 4.11. Relative proportion of vegetation type categories recorded from 221 *Anopheles*-positive larval dipping collections 158

Figure 4.12. Relative proportion of vegetation categories associated with each species 158

Figure 4.13. Proportion of larval dipping collections in which each of the dominant types of emergent vegetation was present for each species separately and for all *Anopheles* larvae combined 160

Figure 4.14. Proportion of larval dipping collections in which each of the dominant types of floating vegetation was present for each species separately and for all *Anopheles* larvae combined 160

Chapter One:
Introduction to *Anopheles* systematics

1.1. Introduction to Mosquitoes (Diptera: Culicidae)

Mosquitoes are common insects throughout the world, well known for their potential to be pests and vectors of disease to humans and other animals because the adult females of most species require a blood meal to develop their eggs. While adult females feed on their hosts, salivary gland fluid containing compounds such as anticoagulants, antiplatelets, and vasodilators, and occasionally pathogenic organisms, is injected into the host while the mosquito imbibes her blood meal (Ribeiro and Francischetti 2003).

Mosquitoes are involved in the transmission of a wide variety of diseases, most notably human malaria, which is spread by *Anopheles* mosquitoes primarily in tropical regions. The World Health Organization estimates that 243 million clinical cases of malaria occurred in 2008, leading to approximately 863,000 deaths (WHO 2009). In Canada, mosquitoes are potential vectors of viruses known to cause at least 5 human and animal diseases, including the Western Equine, Eastern Equine, California, and St. Louis encephalitis viruses (Calisher 1994), as well as the West Nile virus (Turell *et al.* 2001). Although malaria has been considered eradicated from Canada since the 1950s, competent malaria vectors are present in southern Canada in many densely populated regions, and current trends in climate change, international trade, and travel may lead to sporadic local transmission of the disease in the future (Berrang-Ford *et al.* 2010). When outbreaks of known or emerging diseases occur, the key to successful mosquito control is the ability to identify vector species accurately, so that resources can be targeted in the correct areas.

Mosquitoes are members of the family Culicidae in the insect Order Diptera, or "true flies". They have two wings, long legs, and their elongate mouthparts form their most distinctive feature, called the proboscis (Figure 1.1). While only females use it to pierce the flesh of their hosts for a blood meal, both males and females use their proboscis to obtain water as well as floral nectar, an important energy source for both sexes (Clements 1992). Like other dipterans, the culicid life cycle involves complete

metamorphosis, a process with four main stages of development: egg, larva, pupa, and adult (Wood *et al.* 1979; Clements 1992). Eggs (Figure 1.2) are laid on the surface of standing water, or on moist soil that will eventually flood (such as the sides of roadside ditches and along the edges of ponds and marshes), and require contact with standing water in order to hatch (Wood *et al.* 1979). Embryonic development is completed within the egg and, after stimulated to hatch, the legless larva emerges into the water and begins to feed on particulate matter. Mosquito larvae (Figure 1.3) have elaborate mouthparts, which include a pair of labral brushes that they use to create a current and draw water containing food particles towards their mouth (Wood *et al.* 1979).

Larvae swim through the water using side-to-side movements of their abdomen and development proceeds through four progressively larger instar stages (Wood *et al.* 1979). After the fourth instar stage is complete, larvae then transform into pupae (Figure 1.4), the stage during which the mosquito does not feed and the adult form of the mosquito develops inside the pupal casing. When development is complete, the adult splits the pupal casing open and works its way out, standing on the surface of the water and eventually flying away in search of food and a mate (Clements 1992).

In Canada, mosquitoes develop in a wide variety of larval habitats, including forest pools, ponds and marshes, rock pools and tree holes, as well as artificial containers such as rain barrels and used tires. They employ various life cycle and overwintering strategies as well. For example, the eggs of many species of *Aedes* mosquitoes are laid in moist soil in areas that flood when the snow melts each spring, stimulating the eggs to hatch, and when the adults emerge, they mate, the females blood feed, lay their eggs in moist soil, and soon die (Wood *et al.* 1979). The eggs remain dormant until the next year when the snow melts and the cycle begins again.

Most *Anopheles* mosquitoes overwinter as mated, but non-bloodfed, adult females in dark, moist environments (such as old basements, hollow trees and woodpiles), and, each spring, they emerge from their overwintering sites to obtain a blood meal, and lay their eggs on the surface of habitats such as ponds, marshes, and ditches (Wood *et al.* 1979). The larvae hatch and develop into adults, repeating this cycle multiple times throughout the season, and, as the end of the season nears, the last females

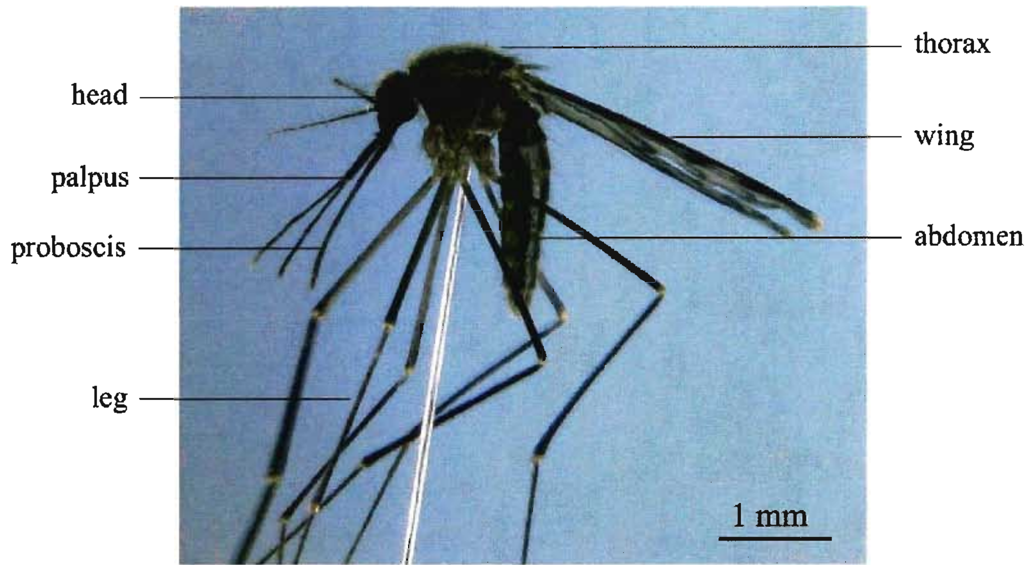

Figure 1.1. Lateral view of an adult female mosquito, *Anopheles earlei.* Two wings extend posteriorly, and six legs ventrally, from the thorax. The proboscis can be seen below two elongate palpi, characteristic of *Anopheles* females.

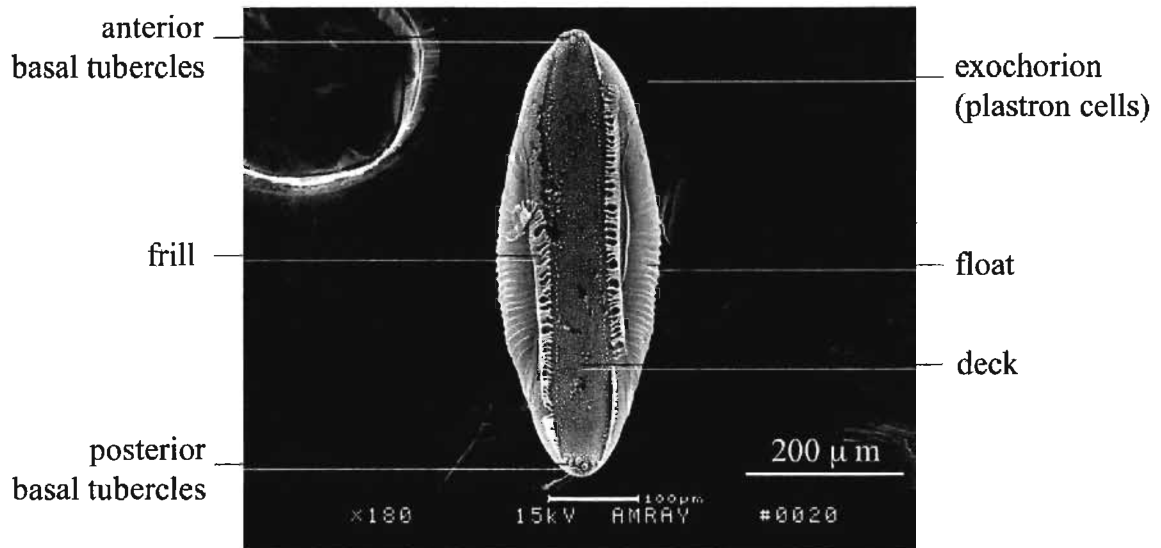

Figure 1.2. Scanning electron micrograph of the ventral surface of a mosquito egg, *Anopheles punctipennis* (chorionic pattern more visible from dorsal aspect). Magnification x180.

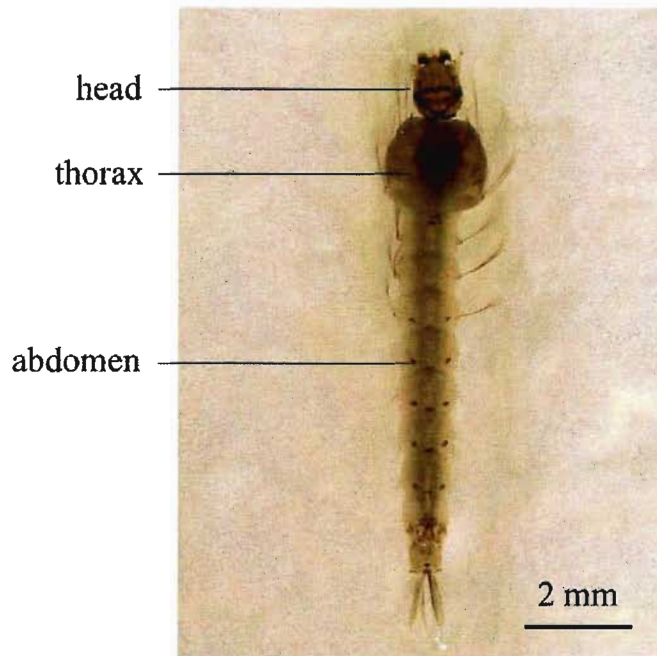

Figure 1.3. Dorsal view of a fourth instar larva, *Anopheles earlei*.

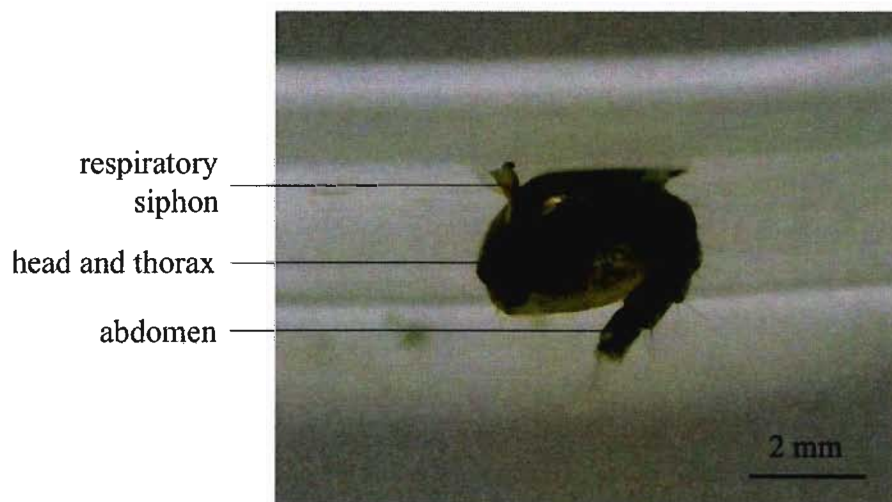

Figure 1.4. Lateral view of a mosquito pupa, *Anopheles punctipennis*. (Note: reflection of the dorsal surface of pupa from the water's surface can be seen above the pupa).

to emerge mate and then locate an overwintering site where they remain mostly dormant until the following year (Wood *et al.* 1979).

These are but a few of the many ways in which mosquitoes have adapted to the Canadian environment. Prior to the current study, eighty-two species in 10 genera of mosquitoes were known from Canada, which exist in diverse environments across the country, from the tiny pitcher-plant mosquito, *Wyeomyia smithii*, whose larvae live in the water that collects inside the pitcher-shaped leaves of the *Sarracenia purpurea* plant found in northern bogs, to the extremely large livestock mosquito, *Psorofora ciliata*, biters so voracious they are reputed to bite through heavy clothing (Wood *et al.* 1979; Thielman and Hunter 2007). Mosquitoes exhibit a wide variety of mating strategies, ecological associations and feeding behaviours, and occur throughout the country. Despite their potential to spread disease and reach population levels high enough to warrant mosquito abatement programs, surprisingly little is known about most mosquito species in Canada.

1.2. Mosquito Systematics

Systematics is the study of biological diversity and evolutionary relationships among organisms, extinct and extant, and is used to construct taxonomic groups (Brooker *et al.* 2010). It involves the discovery, naming and description of species and the development of hypotheses of the phylogenetic relationships among them. Traditionally, systematic investigations of mosquitoes have been based primarily on morphological data, but other types of data, including ecological, behavioural, and now molecular data, are also commonly used. Non-morphological data are particularly important when examining cryptic species complexes, i.e., groups of closely related but reproductively isolated species that are difficult or impossible to identify based on morphological characters (Collins and Paskewitz 1996). The discovery that isomorphic members of species complexes often varied in their vector potential led to rapid advances in the field of mosquito systematics, and alternative (non-morphological) methods of species identification were, therefore, developed (reviewed in Munstermann 1995; Krzywinski and Besansky 2003; Walton *et al.* 1999b; Munstermann and Conn 1997). This is

particularly true within the genus *Anopheles* because many of the vectors of human malaria belong to such cryptic species complexes, whose isomorphic members exhibit behavioural and/or ecological differences that affect their vectorial capacity (Collins and Paskewitz 1996; Besansky 1999).

Anopheline systematists usually include data from at least two types of analyses including, but not limited to, morphological (egg, larval, pupal, or adult characters), genetic (hybridization experiments), cytological (polytene chromosome banding patterns), biochemical (cuticular hydrocarbon, allozyme data), molecular (DNA sequences), and ecological (larval habitat, host associations, etc.). Combinations of data have been used successfully to identify new species, elucidate cryptic species, and propose evolutionary relationships among species (reviewed in Krzywinski and Besansky 2003, Munstermann and Conn 1997; for examples see Reinert *et al.* 1997, Lounibos *et al.* 1998, Nicolescu *et al.* 2004, Reidenbach *et al.* 2009).

Classical mosquito taxonomy was based primarily on morphological data until well into the 20th century. The discovery of polytene chromosomes in *Anopheles* mosquitoes during the 1940s and that they could distinguish among malaria vector and non-vector species in the *Anopheles maculipennis* complex (Frizzi 1947), led to the discovery of other important anopheline species complexes (Coluzzi *et al.* 1977; Foley and Bryan 1991; Ramirez and Desson 2000). Polytene chromosomes are also a source of phylogenetic information, as closely related species can often be differentiated, and the relationships among them determined, based upon chromosomal inversions (White 1978; Pape 1992). However, preparation of the chromosomes for analysis is labour intensive, requires considerable expertise, and is applicable only to the larval and adult female stages. Over the last few decades, molecular data generated by polymerase chain reaction (PCR)-based methods have proven to be valuable species identification and phylogenetic tools. This has led to their increased use in taxonomic and phylogenetic investigations of mosquitoes, particularly within the Anophelinae (Chen *et al.* 2003; Paredes-Esquival *et al.* 2009; Sallum *et al.* 2002; Sallum *et al.* 2007; Sharpe *et al.* 2000; Wilkerson *et al.* 2005).

Apart from major malaria vectors, the systematics of *Anopheles* mosquitoes remained largely at the alpha taxonomy level (i.e., the discovery and naming of new

species) until the end of the 20th century (Krzywinski and Besansky 2003). However, recent studies have sought to understand the higher-level systematics of mosquitoes, including the Anophelinae (Harbach and Kitching 1998; Mitchell *et al.* 2002; Reidenbach *et al.* 2009). In a phylogeny based on morphological characters, Harbach and Kitching (1998) found that the Anophelinae are monophyletic and basal within Culicidae, similar to relationships suggested by the complete coding sequences of the mitochondrial genes cytochrome *c* oxidase I and II (i.e., COI and COII) (Mitchell *et al.* 2002). The results of a recent analysis using morphological and molecular data (i.e., six nuclear protein coding genes) (Reidenbach *et al.* 2009) also agree with those of the previous studies. While these studies include anophelines from outside the Nearctic region, the relationships among Nearctic *Anopheles* species were proposed based on the D2 variable region of 28S ribosomal RNA, a phylogeny that includes most of species that occur in Canada and were examined in this study (Figure 1.5) (Porter and Collins 1996).

Taxonomically, the *Anopheles* mosquitoes are members of the dipteran family Culicidae, which is divided into two main lineages called subfamilies, the Anophelinae and the Culicinae (Harbach and Kitching 1998). The subfamily Anophelinae includes three genera: *Anopheles*, *Bironella*, and *Chagasia* (Harbach and Kitching 2005). Most anopheline species are assigned the genus *Anopheles*, which includes six subgenera: *Anopheles*, *Cellia*, *Kerteszia*, *Lophopodomyia*, *Nyssorynchus*, and *Stethomyia* (Harbach and Kitching 2005). Harbach (2004) published a working hypothesis for the classification and phylogenetic relationships of the genus *Anopheles*, including the subgenus *Anopheles*, to which all Canadian anopheline species belong. Harbach (2004) attempted to place species into increasingly smaller groups, from Subgenera to Section, then to Series, Group and, finally, to Complex and Species, where each taxonomic grouping is believed to be phylogenetically related based on morphological and genetic data. All Canadian anopheline species are members of the Anopheles Series of the Angusticorn Section of the Subgenus *Anopheles*, and within the Anopheles Series, they are further subdivided into the Maculipennis, Plumbeus, and Punctipennis Groups (Figure 1.6) (Harbach 2004).

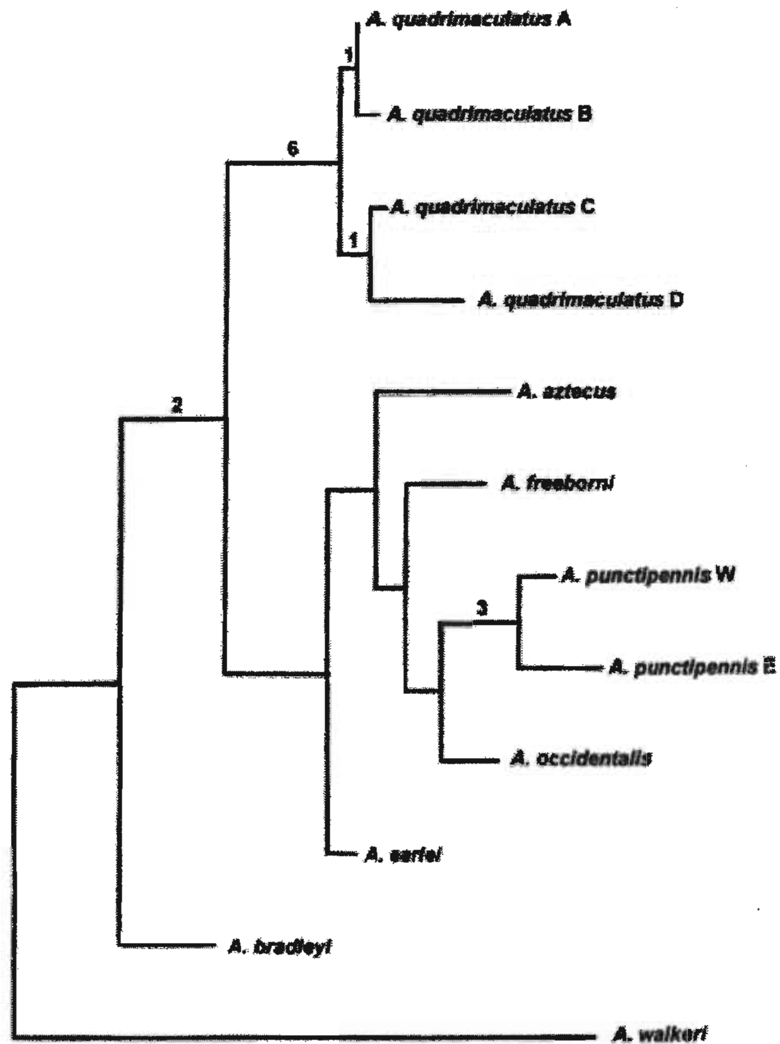

Figure 1.5. Phylogeny of Nearctic *Anopheles* based on D2 region of 28S rRNA (Fig. 3 of Porter and Collins 1996). Note that *Anopheles occidentalis* and *An. aztecus* do not occur in Canada. *Anopheles bradleyi* is a member of the *An. crucians* complex, and while *An. crucians s.l.* has been collected in southern Ontario (Thielman and Hunter 2007), no specimens were collected during the current study. Furthermore, *An. barberi*, which is present in Canada, was not included in the analyses of Porter and Collins (1996).

Subgenus *Anopheles*
- Angusticorn Section
  - Anopheles Series
    - Maculipennis Group
      - *walkeri*
      - Quadrimaculatus Subgroup
        - *quadrimaculatus*
        - *smaragdinus**
      - Freeborni Subgroup
        - *earlei*
        - *freeborni*
        - *occidentalis**
    - Punctipennis Group
      - *perplexens**
      - *punctipennis*
      - Crucians Complex
        - *crucians*
    - Plumbeus Group
      - *barberi*

Figure 1.6. Proposed classification of *Anopheles* mosquitoes from Canada. Groupings represent increasingly related species (adapted from Harbach 2004). * indicates species suspected to occur in Canada based on preliminary morphological identifications or close proximity of known distribution ranges to the Canadian border.

There is a major difference between the phylogenetic tree based on the D2 fragment of 28S rDNA (Porter and Collins 1996) and the relationships among species present in Canada based on morphological and molecular data as proposed by Harbach (2004). Based on D2, *An. punctipennis* (E and W forms) are more closely related to *An. freeborni* and *An. earlei* (Porter and Collins 1996), followed by the *An. quadrimaculatus* species complex, and then *An. walkeri* (Figure 1.5). However, Harbach (2004) places *An. punctipennis* in their own Group, outside the Maculipennis Group, which includes *An. earlei*, *An. freeborni*, *An. quadrimaculatus s.l.*, and *An. walkeri* (Figure 1.6). Cywinska *et al.* (2006) examined a portion of the COI gene in four species of *Anopheles* mosquitoes from Ontario and found that *An. earlei* and *An. punctipennis* sequences were most similar, followed by *An. quadrimaculatus s.s.*, then *An. walkeri*, similar to the results of Porter and Collins (1996). Therefore, there is an apparent incongruence between phylogenies based on molecular data and those that include morphological data, which demonstrates the importance of using multiple types of data in the analysis of *Anopheles* mosquitoes.

While many taxonomic and phylogenetic investigations include some of the anophelines present in Canada, none have included them all. In addition, while many species are now recognized as complexes of isomorphic species, particularly in regions where malaria or other important diseases occur, only two studies have attempted to examine the cryptic species status of species that are of relevance to the current thesis (Wilkerson *et al.* 2004, Reinert *et al.* 1997), but they did not include any specimens that were collected from Canada. Cywinska *et al.* (2006) examined the barcoding region of COI to assess its ability to identify mosquito species present in Canada, but included specimens collected only from Ontario, and many species have much broader distribution ranges. Therefore, cryptic species status has not yet been examined in the *Anopheles* mosquitoes of Canada, despite the medical and veterinary importance of these potential disease vectors near the northern limits of their ranges.

1.3. Species Complexes and Cryptic Species

Many terms are used when describing groups of closely related and/or cryptic species, which may or may not be distinguishable on the basis of morphology alone. The isomorphic nature of such species occurs because speciation does not always involve morphological change (Bickford *et al.* 2006). Authors sometimes use these terms interchangeably and their definitions, as used in this thesis, are provided here for clarification. Cryptic species refer to two or more distinct species that are or were described as a single species because they are difficult or impossible to identify based on morphology (Bickford *et al.* 2006). Sibling species are cryptic sister species, i.e., each other's closest relatives which are difficult or impossible to distinguish morphologically (Bickford *et al.* 2006). The term species complex is used to describe groups of closely related species, and is usually used to describe more recently derived species that have few if any morphological differences among the members of the complex, such as the *Anopheles quadrimaculatus* Complex (Reinert *et al.* 1997). The term species group is used to describe groups of species whose members are more distantly related and often at least some of the members are distinguishable based on morphological characters, such as the Nearctic members of the *Anopheles maculipennis* Group (Porter and Collins 1996).

Cryptic species complexes are common in mosquitoes (Walton *et al.* 1999b), including the genus *Anopheles* (Collins and Paskewitz 1996). Because the members of anopheline species complexes often differ in ecological or behavioural characteristics that affect their ability to transmit disease (Collins and Paskewitz 1996), the ability to accurately identify cryptic species is essential for implementing effective vector control. This is best illustrated using the "Anophelism without malaria" problem that hindered control of the disease in Europe during the early 1900s until it was discovered that the primary vector, *An. maculipennis,* was actually a group of isomorphic species (i.e., they had been dealing with *An. maculipennis s.l.*), whose members differed in their potential to transmit *Plasmodium* parasites, the causative agents of malaria (Walton *et al.* 1999). Since then, particularly in regions where malaria is endemic, many cryptic species

complexes have been discovered within the genus *Anopheles* (Collins and Paskewitz 1996; Besansky 1999).

The first reliable method for identifying cryptic species involved the use of polytene chromosomes (Collins and Paskewitz 1996). Polytene chromosomes are found in specialized cells (the ovarian nurse cells of adult females and salivary gland cells of fourth instar larvae) that are formed because the cells undergo repeated replication of the chromosomes without cell division, a process called endoreduplication (Clements 1992). This results in giant chromosomes with discernible banding patterns that can be used to distinguish among even closely related and/or isomorphic species (Clements 1992). Frizzi (1947) first used polytene chromosomes to elucidate members of the *Anopheles maculipennis* complex in Europe, and Coluzzi *et al.* (1977) for the members of the *Anopheles gambiae* complex in Africa, both of which contain major malaria vectors in the regions where they occur. Since their discovery, not only have polytene chromosomes been used to identify the cryptic members of many species complexes, they can be an important source of phylogenetic information and can be used to investigate population structure and speciation processes (Guelbeogo *et al.* 2005).

The polytene chromosome method of elucidating cryptic species has a number of disadvantages. Preparation of the chromosomes and interpretation of the banding patterns are labour intensive and require considerable expertise (Collins and Paskewitz 1996; A. Thielman, *pers. obs.*). Also, not all anophelines have discernible polytene chromosome patterns and not all anopheline sibling species have detectable differences in banding pattern (Krzywinski and Besansky 2003). This method is also applicable only to the larval and adult female stages (Krzywinski and Besansky 2003). Therefore, cytogenetic studies, once the most widely used technique for identifying cryptic anopheline species, are being replaced by molecular methods (Collins and Paskewitz 1996).

The first molecular methods of cryptic species identification involved protein electrophoresis of enzyme variants, or allozymes (Krzywinski and Besansky 2003), however, recently derived species do not always differ in allozyme profiles and proper preservation of material for such studies is often difficult (Walton *et al.* 1999). DNA-based methods using PCR have many advantages over morphological, cytological and

biochemical methods, and have contributed to their almost universal use in studies of insect systematics today. DNA offers almost unlimited polymorphic markers and is easily preserved (Krzywinski and Besansky 2003). Only a minute amount of DNA, such as one leg (or part of a leg for larger insects) is required, allowing specimens to be retained for morphological vouchers, used in other types of analyses, or kept alive for crossing experiments (Krzywinski and Besansky 2003). Unlike protein, DNA is always detectable and it provides more sources of variation since it measures changes at the nucleotide level (Behura 2006).

The success of molecular methods that use mitochondrial (mtDNA) and ribosomal DNA (rDNA) sequences to identify and elucidate cryptic anopheline species (Lunt *et al.* 1996; Collins and Paskewitz 1996) has led to their widespread use in studies of *Anopheles* systematics, and in the discovery of new species and species complexes (Porter and Collins 1991; Paskewitz *et al.* 1993; Cornel *et al.* 1996; Hackett *et al.* 2000; Foley *et al.* 2007; Paredes-Esquivel *et al.* 2009).

1.4. Ecological Associations

The importance of ecological traits in investigations of *Anopheles* systematics has long since been recognized (Kitzmiller 1959), and remains so for many reasons. Ecological associations, such as host feeding associations (e.g., humans vs. other animals) and larval habitat association (e.g., permanent vs. temporary pools), have a direct influence on disease transmission and are, therefore, important from a human health perspective. Many studies have attempted to characterize the larval habitat of *Anopheles* species (e.g., habitat size, seasonal prevalence, and presence of aquatic vegetation) to better understand the ecology of vector species and improve larval control measures in disease endemic regions (for examples, see Rejmankova *et al.* 1993; Manguin *et al.* 1996; Gimnig *et al.* 2001; Shililu *et al.* 2003). Coluzzi *et al.* (1979) proposed that speciation in the *An. gambiae* complex was related to adaptation to human-induced environmental changes, and recent studies suggest that speciation in this medically important species complex may be due niche expansion (Constantini *et al.* 2009; Gimonneau *et al.* 2010).

The ecological hypothesis of speciation has been recognized since the 1940s and was largely accepted by evolutionary biologists despite a lack of empirical evidence for its occurrence in nature (Schluter 2001). However, recent studies have begun to provide this evidence (McKinnon *et al.* 2004; Langerhans *et al.* 2007; Schluter 2009). Since evidence for ecological speciation exists, and the distribution of many anopheline species is related to ecological factors (e.g., larval habitat characteristics), the examination of ecological traits is important in investigations of cryptic species. For example, once species of the *An. gambiae* complex were identifiable based on polytene chromosome and hybridization data, the ecological differences among species were discovered, with larvae of *An. gambiae s.s.* and *An. arabiensis* present in fresh water habitats and those of *An. melas* and *An. merus* occurring in coastal salt water ones (Coluzzi *et al.* 1977). Characterization of larval habitats associated with vector and non-vector species can improve the accuracy and efficiency of mosquito control efforts when required.

Another example of ecological differences associated with cryptic species involves the larval habitat of the morphologically similar species *An. punctipennis* and *An. perplexens*. Larvae of *An. punctipennis* occur in a wide variety of temporary and permanent habitats including marshes, ponds, forest pools, rock pools, and artificial containers (Carpenter and LaCasse 1995; Wood *et al.* 1979), but those of *An. perplexens* are restricted to the highly calcareous water characteristic of limestone springs present throughout the type locality in southern Georgia, USA (Bellamy 1956). These cryptic taxa are considered good species based on polytene chromosome and hybridization data (Kreutzer and Kitzmiller 1971a; Kreutzer and Kitzmiller 1971a), but larval and adult morphological characters are unreliable (Bellamy 1956; Fritz *et al.* 1991). Studies of cryptic frog species in limestone habitats include the description of a new species from limestone forests in the Philippines, a habitat from which 28 cryptic species are now known from only three morphological species groups (Siler *et al.* 2010). Other *Anopheles* species are known from limestone habitats as well, including a new species, *An. cucphuongensis*, collected from limestone rock pools in Vietnam (Phan *et al.* 1990). Therefore, the analysis of ecological data, such as larval habitat associations, represents an important perspective from which to study *Anopheles* systematics.

1.5. Integrated approach to *Anopheles* systematics

Mosquito taxonomy has traditionally been established based on morphology. Egg, larval, pupal, and adult characters are used in the development of dichotomous keys that allow identification to the species level. However, because cryptic species are common in mosquitoes, morphology alone is often insufficient to correctly identify the members of such groups. This is particularly important in *Anopheles* mosquitoes because successful control of cryptic vector species depends on their accurate identification. The members of cryptic species complexes don't necessarily differ in all possible ways (i.e., cytological, biochemical, ecological, and molecular). Therefore, a multidisciplinary approach to *Anopheles* systematics that integrates data from as many sources as possible is required to address the complexity that is often present among anophelines (Besansky 1999), as well as increase the accuracy of hypotheses of phylogenetic relationships.

Although morphology is often not enough when dealing with cryptic species, it remains the simplest, fastest, and least expensive method of identification when possible (White 1977). As quoted by leading *Anopheles* systematist Ralph Harbach when discussing its importance, "...morphology is a prerequisite for identifying species to complex or group prior to the application of molecular and other methods. You need to get into the ball park before you can play the game" (Krzywinski and Besansky 2003, p. 115). Also, once cryptic species are discovered based on other types of data (e.g., DNA sequence data), previously unrecognized minor morphological differences are sometimes discovered upon morphological comparisons of the resulting groups. Sometimes cryptic anophelines differ morphologically only in certain life stages (Linley and Kaiser 1994). Therefore, examination of morphological data is essential to investigations of *Anopheles* systematics.

When morphological data alone are insufficient to identify cryptic species, other types of data (e.g., cytological, ecological, molecular) are required. While cytological data (i.e., polytene chromosome banding patterns) have successfully revealed the presence of many cryptic anopheline species, it is a labour-intensive technique and applicable only certain life stages, and, therefore, not ideal for routine species identification. Molecular data, particularly COI and ITS2 sequences, have been used

successfully to identify species and elucidate cryptic species, and their use has become standard in studies of *Anopheles* systematics. Since cryptic species often occupy different ecological niches, and the knowledge of larval habitats is important with respect to disease control, ecological data are often also examined. However, it is the use of multiple types of data that often provides the most insight when investigating the systematics of *Anopheles* mosquitoes (Reinert *et al.* 1997; Lounibos *et al.* 1998). The importance of an integrated approach has been recognized for decades (Faran 1979), as it can contribute significantly to the ability to accurately identify potential cryptic vectors of disease, and, therefore, can allow the correct species to be targeted in mosquito control programs when necessary.

1.6. *Anopheles* species known from Canada

Although malaria has not been present in Canada since the 1950s, native *Anopheles* species were involved in the transmission of human malaria in Upper Canada during the 1800s (Zucker 1996; Wood *et al.* 1979). Today, *Anopheles* species are potential vectors of the filarial worm *Dirofilaria immitis* that causes dog heartworm (Kartman 1953), the West Nile (WNv) virus (Turell *et al.* 2001), and other viruses (Calisher 1994) which can cause severe illness and death in humans, and recent studies have shown that malaria could recur in Canada in the future (Berrang-Ford *et al.* 2009). This thesis represents the first attempt to determine if cryptic species complexes are present among the traditionally recognized species of *Anopheles* mosquitoes in Canada, and to describe the morphological, molecular, and ecological differences among them.

In Canada, most anophelines lay their eggs singly on the surface of standing water, usually permanent or semi-permanent ponds, marshes, sloughs and ditches with emergent aquatic vegetation (Wood *et al.* 1979). *Anopheles* larvae live at the surface of the water where they feed (Wood *et al.* 1979), usually resting on top of floating plant material, especially algae (A. Thielman, *pers. obs.*). Larval development usually takes a couple of weeks, undergoing multiple generations throughout the summer and, in autumn, non-bloodfed adult females locate hibernacula, overwintering in damp, dark places such as caves, hollow trees and unheated buildings (Wood *et al.* 1979). In early

spring, the females emerge and seek out a blood meal to begin the cycle over again. Host-seeking females are usually active from early evening and well into the night, and they can produce many offspring in their lifetime, up to 5 or more batches of ~100-200 eggs (Wood *et al.* 1979).

The mosquito genus *Anopheles* is one of 10 genera, and includes seven of the 82 species, known to occur in Canada (Thielman and Hunter 2007). Native species include *Anopheles barberi* Coquillett, *An. earlei* Vargas, *An. freeborni* Aitken, *An. punctipennis* (Say), *An. quadrimaculatus sensu lato* Say, and *An. walkeri* Theobald (Wood *et al.* 1979), as well as *An. crucians sensu lato* Wiedermann, a relatively recent record for Canada that was collected from locations in Windsor/Essex County in 2002 and 2003 through West Nile virus (WNv) mosquito surveillance conducted at Brock University (Thielman and Hunter 2007).

Therefore, to provide background information and a context for the investigation of cryptic species status among *Anopheles* species in Canada, a brief description of each anopheline known in Canada is provided herein.

1.6.1. *Anopheles barberi* Coquillett, 1903

Anopheles barberi is a small mosquito, distinct from the other *Anopheles* species in Canada in many ways. Larvae of *An. barberi* have been found primarily in tree holes in Point Pelee National Park (Smith and Trimble 1973) and around Perth, Ontario (Wood *et al.* 1979), but likely occur throughout southern Ontario wherever suitable habitat exists (Figure 1.7). A population was recently discovered in Niagara Falls, Ontario, in used tires that were placed in a woodlot by a colleague to study larval competition among *Aedes* mosquito species. *Anopheles barberi* is the only anopheline species in Canada that is included in the Plumbeus Subgroup (Figure 1.6) (Harbach 2004), and overwinters in the larval stage, likely as second instars (Wood *et al.* 1979). Copeland (1987) found that laboratory colonies were easily established if sufficient larvae were present to create the first generation. Fourth instar larvae are predacious on small mosquito larvae, can consume large numbers of first instars (Peterson *et al.* 1969), and have been observed to feed on both conspecific and heterospecific larvae (A. Thielman, *pers. obs.*).

1.6.2. *Anopheles crucians sensu lato* Wiedermann, 1828

Anopheles crucians s.l. was first discovered in Canada during the WNv mosquito surveillance program at Brock University. One female was collected in Point Pelee Provincial Park in Leamington, Ontario in 2002 and another from the Toronto Zoo in 2003 (Thielman and Hunter 2007). Its previous known distribution in the USA includes most southern states with northern limits recorded as Indiana, Illinois, Ohio, and the southern regions of Pennsylvania and New York (Figure 1.8) (Darsie and Ward 2005). *An. crucians s.s.* is the type member of a species complex that includes two other named species (i.e., *An. bradleyi* and *An. georgianus*), as well as three previously unrecognized, and (as of 2011) still undescribed, species (Wilkerson *et al.* 2004).

Since the specimens collected from Canada were identified based only on morphology (i.e., subcostal pale-scaled spot greatly reduced, not including the costal wing vein), they can be identified only as *An. crucians s.l.* The larval habitat of *An. crucians s.l.* includes permanent or semi-permanent ponds, swamps, and lake edges, and they are most abundant in the acidic water of cypress swamps in Florida and Georgia (Floore *et al.* 1976). Adults feed primarily on mammals, including humans, and usually feed outdoors at dusk; however, they do occasionally enter houses or bite during the day, especially on cloudy days or in the shade (Carpenter and LaCasse 1955).

1.6.3. *Anopheles earlei* Vargas, 1943

Anopheles maculipennis Hearle, 1927
Anopheles maculipennis occidentalis Aitken, 1945

Anopheles earlei is present throughout Canada below the tree line, from Nova Scotia to British Columbia, and as far north as Churchill, Manitoba and northern Yukon (Figure 1.9) (Wood *et al.* 1979). Adult females are known to overwinter in many natural habitats such as mammal burrows, hollow logs and trees, and beaver lodges, as well as in buildings (Wood *et al.* 1979). They emerge on the first warm days of spring to seek out their first blood meal, and are probably capable of multiple generations throughout the season in more southern regions, but are likely univoltine in the northern part of their range (Wood *et al.* 1979). Larvae of *An. earlei* are usually found in cold, clear water in

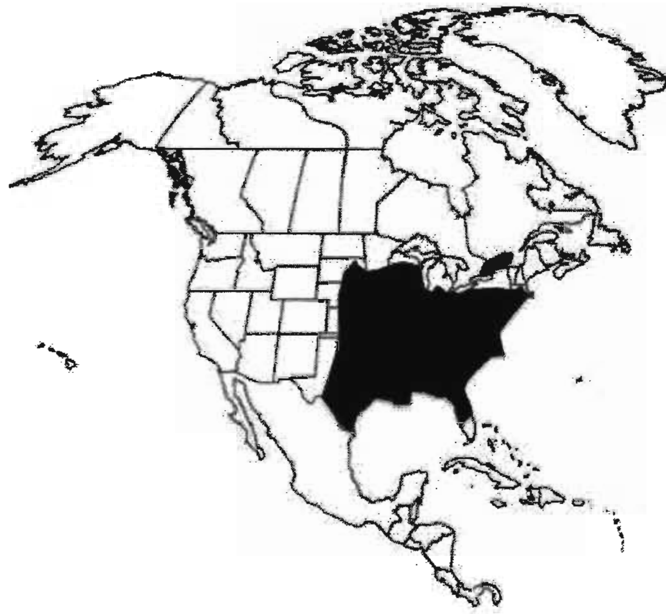

Figure 1.7. Geographic distribution of *Anopheles barberi* in North America. Shaded area represents known range (adapted from Darsie and Ward 2005).

Figure 1.8. Geographic distribution of *Anopheles crucians sensu lato* in North America. Shaded area represents known range and dots known collection locations (adapted from Darsie and Ward 2005).

the shallow margins of permanent and semi-permanent ponds with emergent and floating vegetation and occasionally in woodland pools and marshes (Carpenter and LaCasse 1955). *Anopheles earlei* females usually bite at dusk and shortly after dark, but are known to enter homes and feed during the night (Carpenter and LaCasse 1955).

1.6.4. *Anopheles freeborni* Aitken, 1939

Anopheles maculipennis spp. *freeborni* Aitken, 1939
Anopheles quadrimaculatus Hearle 1927 and many authors before 1939

The distributional range of *An. freeborni* includes most of the western USA, extending northward into Canada only in British Columbia (Figure 1.10) (Darsie and Ward 2005). Adult females overwinter in a variety of locations including abandoned buildings and mines, root cellars, animal burrows, and rock piles (Wood *et al.* 1979). *Anopheles freeborni* females emerge in early spring to seek out a blood meal and larvae are usually found in pools and sloughs formed by creeks, large marshes and irrigation pastures (Wood *et al.* 1979). The females are most active at dusk and throughout the night, entering houses readily to feed on humans (Carpenter and LaCasse 1955).

1.6.5. *Anopheles punctipennis* (Say, 1823)

Culex punctipennis Say, 1823
Culex hyemalis Fitch, 1847
Anopheles stonei Vargas, 1941

Anopheles punctipennis occurs across the southern parts of Canada with its range extending north from the USA into British Columbia, Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia (Darsie and Ward 2005) (Figure 1.11). Non-bloodfed females overwinter in sites similar to those listed for *An. earlei* and *An. freeborni,* and all three species often occur together in regions where their ranges overlap (Wood *et al.* 1979). Adults emerge in early spring to seek a blood meal and lay eggs, a cycle they likely complete multiple times throughout the season, similar to *An. earlei* and *An. freeborni* (Wood *et al.* 1979).

Larvae are found in a wide variety of habitats including ponds, temporary pools, stream edges, hog wallows, rain barrels, and other artificial containers (Carpenter and

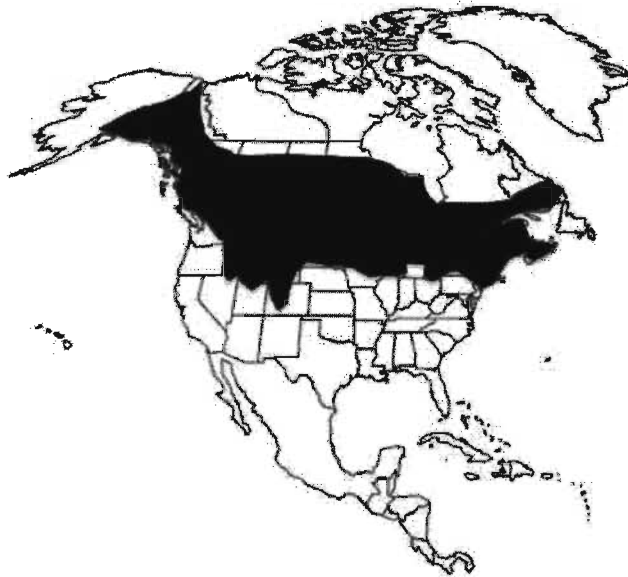

Figure 1.9. Geographic distribution of *Anopheles earlei* in North America. Shaded area represents known range (adapted from Darsie and Ward 2005).

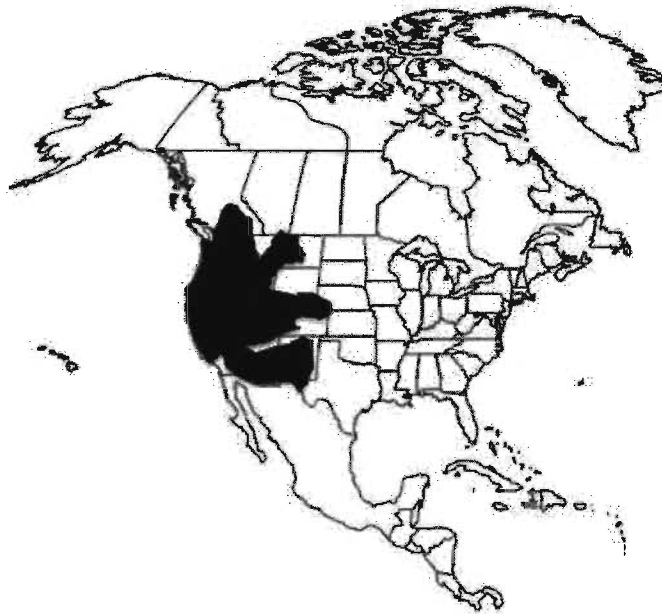

Figure 1.10. Geographic distribution of *Anopheles freeborni* in North America. Shaded area represents known range (adapted from Darsie and Ward 2005).

LaCasse 1955). Females feed mostly at dusk, but will bite during the day if disturbed or in wooded areas (Carpenter and LaCasse 1955). Molecular studies of *An. punctipennis* in the USA have revealed eastern (E) and western (W) forms of this species based on the expansion D2 region of 28S rDNA (Porter and Collins 1996), but examination of COI haplotypes present among samples collected from various locations throughout Connecticut did not reveal evidence of reproductively isolated populations based on geographic barriers or latitude (Fairley *et al.* 2000).

1.6.6. *Anopheles quadrimaculatus sensu stricto* (Say, 1824)

Anopheles quadrimaculatus Say, 1824
Anopheles annulimanus Wulp 1867

Until recently, *An. quadrimaculatus sensu lato* was considered to be a single species throughout its range, from the eastern USA extending northward into southern Ontario and Quebec (Wood *et al.* 1979) (Figure 1.12). In the 1970s, *An. quadrimaculatus s.l.* was the least common anopheline in Canada of the four large "marsh-breeding" species (i.e., *An. earlei, An. punctipennis, An. quadrimaculatus s.l.*, and *An. walkeri*), with very little known about this species in Canada (Wood *et al.* 1979). Females are known to overwinter in hollow trees, cellars, caves, and similar habitats, and they emerge in spring to seek a blood meal and begin the first of many generations for the season (Wood *et al.* 1979).

Larvae are found primarily in permanent fresh water, such as sluggish streams, canals, ponds, and lakes with emergent or floating vegetation (Carpenter and LaCasse 1955). In 1997, Reinert *et al.* described *An. quadrimaculatus* as a complex of five sibling species: *An. quadrimaculatus s.s.*, *An. diluvialus*, *An. inundatus*, *An. maverlius*, and *An. smaragdinus*. Their conclusions were based on multiple types of data, including morphological, cytological, biochemical, molecular, and ecological data (Reinert *et al.* 1997). While the ranges of three of the newly recognized sibling species are believed to be restricted to the southern states, *An. smaragdinus* has an estimated distribution nearly as broad as *An. quadrimaculatus s.s.* (Figure 1.16), extending almost as far north as *An. quadrimaculatus s.s.* (Figure 1.12) (Levine *et al.* 2004).

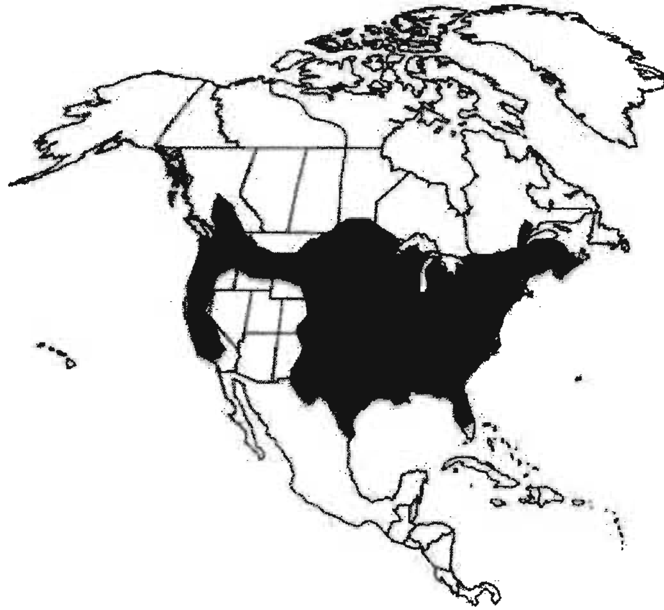

Figure 1.11. Geographic distribution of *Anopheles punctipennis* in North America. Shaded area represents known range (adapted from Darsie and Ward 2005).

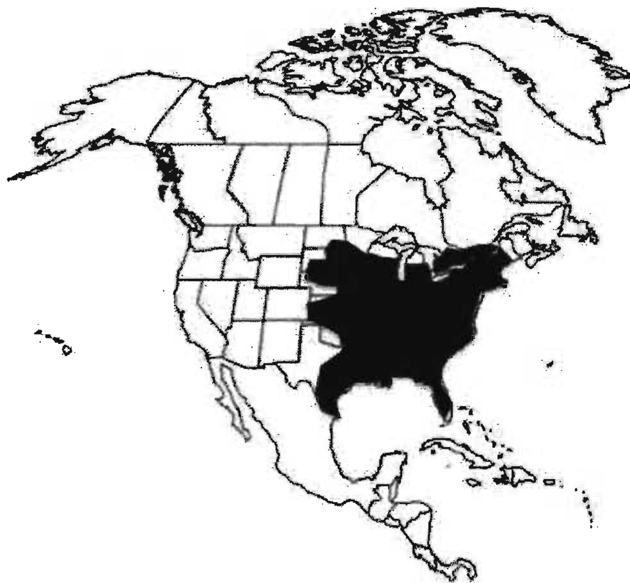

Figure 1.12. Geographic distribution of *Anopheles quadrimaculatus sensu lato* in North America. Shaded area represents known range (adapted from Darsie and Ward 2005).

1.6.7. *Anopheles walkeri* Theobold, 1901

Anopheles walkeri occurs throughout the eastern USA, extending north into Canada to Saskatchewan, Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia (Darsie and Ward 2005) (Figure 1.13). It is the only anopheline species in North America that overwinters in the egg stage, at least in the northern part of its range (Wood *et al.* 1979). Eggs laid at the end of the season are larger and morphologically distinct from those laid earlier in the season and do not hatch in spring unless they experience a lengthy cold period, as in nature during the winter (Wood *et al.* 1979). Larvae of *An. walkeri* are usually found in ponds and marshes with stable water levels overgrown with emergent vegetation (Carpenter and LaCasse 1955; Wood *et al.* 1979). Adults tend to rest on vegetation along shorelines and will bite during the day if their resting site is disturbed (Wood *et al.* 1979), but are also known to enter houses during the night to feed on humans (Carpenter and LaCasse 1955).

1.7. *Anopheles* suspected to occur in Canada

In addition to these seven known species, three species are suspected to occur in Canada: *An. occidentalis* Dyar and Knab, *An. perplexens* Ludlow, and *An. smaragdinus* Reinert. *Anopheles occidentalis* is currently known from the western USA, and although early authors recorded this species from British Columbia, Wood *et al.* (1979) suggests that these specimens were likely misidentified since all the Canadian specimens they examined were the closely related and morphologically similar species, *An. earlei*. *Anopheles perplexens* is currently known from scattered locations throughout the eastern USA, with records approaching southern Ontario. Many of the adult mosquitoes collected from this region through WNv surveillance and the current study exhibited the pale-scaled wing spots greatly reduced in size that are usually characteristic of *An. perplexens* (Bellamy 1956; Darsie and Ward 2005). *Anopheles smaragdinus* is a relatively recently recognized species and member of the *An. quadrimaculatus* sibling species complex. Although three members of the complex are restricted to more southern states, the distribution of *An. smaragdinus* is predicted to approach the Canadian border

(Levine *et al.* 2004), and many specimens collected through the current study keyed out to *An. smaragdinus* based on morphological characters described by Darsie and Ward (2005).

1.7.1. *Anopheles occidentalis* Dyar and Knab, 1906

Anopheles maculipennis occidentalis Aitken, 1945

Anopheles occidentalis is known from the western USA, including California, Oregon, and Washington (Figure 1.14) (Darsie and Ward 2005; Sames *et al.* 2007). Larvae occur in a variety of habitats, such as stagnant creeks, water troughs, and shallow hillside seepages along the Pacific coast, and are often found in the shallow edges of ponds in shade cast by cattails and other aquatic vegetation (Carpenter and LaCasse 1955). Adults remain in the vicinity of the larval habitat and rarely feed on humans (Carpenter and LaCasse 1955). Although all potential specimens of *An. occidentalis* from British Columbia have since been identified as the closely related and morphologically similar native species *An. earlei*, the close proximity of its range to the province warrants more detailed examination of specimens from that region. There is potential for this species order to migrate into Canada in the future should its range expand northward with continued climate change.

1.7.2. *Anopheles perplexens* Ludlow, 1907

Anopheles punctipennis Howard, Dyar and Knab 1917

The species status of *An. perplexens* remained a source of contention for many years. Some specialists believed *An. perplexens* represented a valid species, whereas others considered this entity to represent a morphological variant of *An. punctipennis* (Bellamy 1956). Kreutzer and Kitzmiller (1971a, 1971b) conducted hybridization experiments that revealed sufficient differences in the polytene chromosomes and offspring viability to validate the specific status of *An. perplexens*. SEM studies of egg morphology subsequently revealed minor morphological differences that could be used to distinguish *An. perplexens* from *An. punctipennis* (Linley and Kaiser 1994). *Anopheles perplexens* is known from locations scattered throughout the eastern USA, as far north as Ohio and New York (Figure 1.15) (Darsie and Ward 2005). Larvae of *An. perplexens*

Figure 1.13. Geographic distribution of *Anopheles walkeri* in North America. Shaded area represents known range (adapted from Darsie and Ward 2005).

Figure 1.14. Geographic distribution of *Anopheles occidentalis* in North America. Diamonds represent the known collection locations (adapted from Darsie and Ward 2005; Sames *et al.* 2007).

are found mainly in alvar-type habitats, particularly the limestone sinkholes in Georgia (Bellamy 1956). However, many specimens with wing scale patterns similar to those of *An. perplexens* have been collected throughout Ontario during WNv surveillance. Although this character is known to be variable (Fritz *et al.* 1991), sufficient numbers were found with *An. perplexens*-type wing scale patterns to suggest the possibility that this species occurs in southern Ontario.

1.7.3. *Anopheles smaragdinus* Reinert 1997
Anopheles quadrimaculatus Say 1824

Anopheles smaragdinus is a recently described species in the *An. quadrimaculatus* sibling species complex (Reinert *et al.* 1997). An integrated approach using multiple types of data, including morphological (egg, larva, and adult), cytological (polytene X chromosome), molecular (ITS2 sequence), genetic (hybridization), biochemical (allozyme), and ecological data, revealed the presence of five previously cryptic species in this complex (Reinert *et al.* 1997). *Anopheles quadrimaculatus s.s.* occurs throughout the entire range of the species complex (Figure 1.12), and the distributions of three members of the complex (*An. diluvialus*, *An. inundatus*, and *An. maverlius*) are restricted to southern states such as Florida, Louisiana, and Texas (Levine *et al.* 2004). The distribution of *An. smaragdinus*, however, is predicted to be almost as broad as that of *An. quadrimaculatus s.s.*, occurring as far north as Pennsylvania, Ohio, and Indiana (Figure 1.16) (Levine *et al.* 2004). Larval and adult morphological characters used to distinguish the members of the *An. quadrimaculatus* species complex involve the number and branching of setae, and identification of *An. quadrimaculatus s.l.* specimens collected through the current study using the key of Darsie and Ward (2005) suggested the presence of *An. smaragdinus* in southern Ontario.

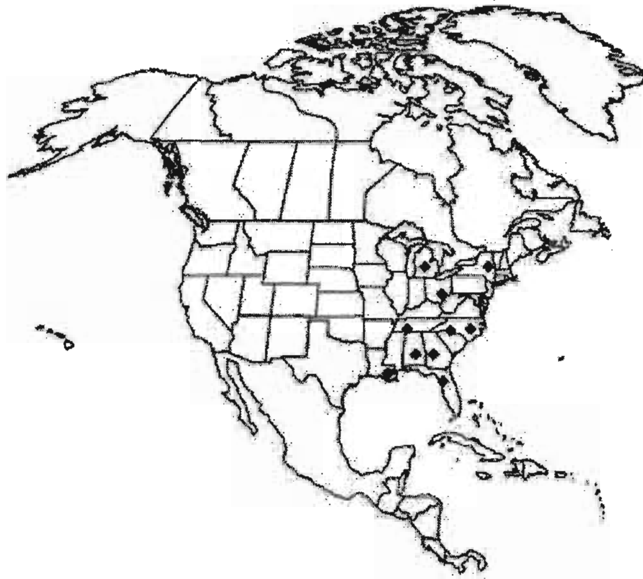

Figure 1.15. Geographic distribution of *Anopheles perplexens* in North America. Diamonds represent known collection locations (adapted from Darsie and Ward 2005).

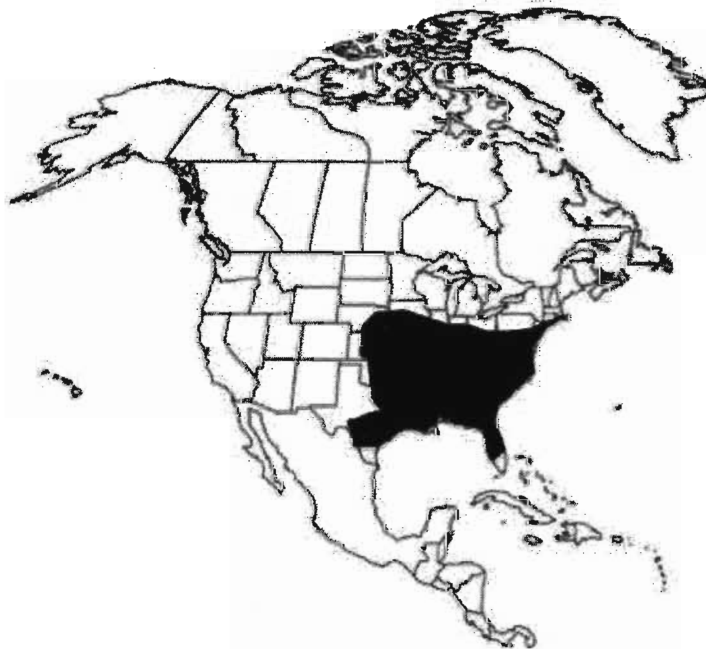

Figure 1.16. Estimated distribution of *Anopheles smaragdinus* in North America. Shaded area represents its predicted range (adapted from Levine *et al.* 2004).

1.8. Status of knowledge in Canada

The greatest source of information regarding the mosquito species of Canada remains the work of Wood *et al.* (1979), the result of years of collection and morphological identification of specimens collected throughout the country. Wood *et al.* (1979) include anatomical illustrations, genus and species level keys, descriptions of species, distribution maps, and bionomic information. The largest collection of Canadian specimens is held in the Canadian National Collection of Insects (CNCI), Agriculture and Agri-Foods Canada, Ottawa, Ontario. While this collection is in the process of being added to a database at the CNCI, it is currently not publicly available online.

With the introduction of West Nile virus to Canada, first detected in southern Ontario in 2001 (Drebot *et al.* 2003), mosquito surveillance programs were first developed in that province, and then established in other provinces as the disease spread. While most of these programs conducted surveillance from June to September, with identification of specimens to species level, they were conducted by pesticide and consulting companies and the results are unpublished. We recently completed a Species at Risk assessment of the mosquito species of Canada for the Canadian Wildlife Service and the Committee on the Status of Endangered Wildlife in Canada (Thielman and Hunter 2010), for which mosquito data were readily provided by some, but not all, provinces and/or territories.

The Species at Risk project required the assignment of general status ranks at both the national and provincial/territorial levels for the mosquito species of Canada and it revealed a considerable lack of knowledge of mosquito biodiversity within Canada, particularly with respect to abundance levels. Although many provincial/territorial species lists were available, very few contained reference to abundance levels other than general descriptions such as "rare", "common", or "abundant". We compared what was known up to and including Wood *et al.* (1979), with any literature published after 1979, and with recent data obtained through West Nile virus surveillance (if available). This resulted in many new provincial/territorial species records and revealed some apparent losses of species from provinces and territories as well. The results of this study can be found on the Wild Species website (www.wildspecies.ca) (Thielman and Hunter 2010).

To date, there has been no attempt to use molecular data to investigate cryptic species of the genus *Anopheles* in Canada using either material in existing collections or newly collected material. Four of the seven species known from Canada have been examined using molecular data to assess the ability of the barcoding region of the COI gene to identify mosquito species in Canada (Cywinska *et al.* 2006). However, the study included only 4-15 specimens per species, included only Ontario material, and did not reveal the presence of cryptic species within the taxa examined (i.e., *An. earlei*, *An. punctipennis*, *An. quadrimaculatus*, and *An. walkeri*) (Cywinska *et al.* 2006).

1.9. Summary

In summary, *Anopheles* mosquitoes are potential vectors of disease and many anopheline species are now recognized as complexes of isomorphic species based on ecological, molecular, and other types of data. Members of species complexes often differ in ecological or behavioural traits, such as larval habitat or host-feeding associations, which influence their ability to transmit pathogens. This, in turn, influences whether they represent a risk to human and animal health. Investigations of the systematics of *Anopheles* mosquitoes are common wherever malaria occurs, but little is known about species that occur near the northern limits of their ranges in Canada, despite their potential for transmitting diseases to humans and animals.

Therefore, my research objective was to examine *Anopheles* mosquitoes using morphological, molecular, and ecological data for evidence of cryptic species within anopheline collections from across Canada.

Chapter Two:
Morphological Analyses of *Anopheles* mosquitoes from Canada

2.1. Introduction

Classical taxonomic investigations of *Anopheles* mosquitoes were based primarily on morphology, which remained the main source of data for the identification of cryptic species until the development of cytological and biochemical methods in the mid-20th century (Collins and Paskewitz 1996). Early studies of systematics were often complemented with ecological, behavioural, and hybridization data, such as larval habitat association (fresh versus brackish water), host feeding preference (birds versus humans), and mating incompatibility (offspring sterility). In recent years, molecular methods of species identification based on mitochondrial (mtDNA) and ribosomal (rDNA) DNA sequence data have contributed substantially to the identification of cryptic *Anopheles* species and are now commonly used to infer phylogenetic relationships (Caterino *et al* 2000; for examples see Wilkerson *et al.* 2005; Foley *et al* 2007; Paredes-Esquivel *et al.* 2009). However, morphological phylogenies provide the foundation upon which studies of evolutionary relationships at and above the species level should be based (Harbach 2004).

Species are often distinguishable in all life stages and sexes (egg, larva, pupa, male and female), but due to a high degree of morphological conservatism within the genus *Anopheles*, some species can only be recognized based on some life stages (White 1977; Krzywinski and Besansky 2003). This is particularly true in the case of cryptic species, which are by definition difficult, if not impossible, to identify based on morphology alone (White 1977). For example, *An. perplexens* is morphologically similar to *An. punctipennis* (Bellamy 1956), and while morphological characters for identifying adults and larvae are known to be variable and unreliable (Bellamy 1956; Fritz *et al.* 1991), these two species can be distinguished reliably on the basis of egg morphology (Linley and Kaiser 1994). Brief descriptions of the life stages of *Anopheles* mosquitoes are provided herein to introduce the morphological component of my thesis research.

2.1.1. Egg Morphology

The taxonomic importance of egg morphology in mosquitoes was first recognized in the early 1900s when microscopic examination of mosquito eggs revealed morphological differences that could be used to identify species of *Anopheles* mosquitoes (White 1977). Egg characters were used in the elucidation of many species complexes and are often among the first morphological changes apparent in recently derived species (Kitzmiller 1959; White 1977). Horsfall (1970) described methods for the examination of egg morphology using scanning electron microscopy (SEM), which allowed minor structural differences between species to be detected. Egg structures commonly used to differentiate anopheline species include deck width, basal tubercles, float configuration, and chorionic patterning (Kitzmiller 1959; White 1977) (Figure 2.1).

Previous SEM studies of the eggs of the two species suspected to occur in Canada, *An. perplexens* and *An. smaragdinus*, revealed structural differences that can be used to distinguish them from their cryptic counterparts (i.e., *An. punctipennis* and *An. quadrimaculatus s.s.*, respectively). The eggs of sibling species *An. punctipennis* and *An. perplexens* differ in width of the deck (Linley and Kaiser 1994) (Figure 2.1). SEM analysis of the eggs of members of the *An. quadrimaculatus* species complex also revealed minor structural differences, which can be used to distinguish *An. smaragdinus* and *An. quadrimaculatus s.s.* (Linley *et al.* 1993) (Figure 2.2).

Although not examined under SEM, the eggs of other anopheline species present in Canada have been described or illustrated in early publications (1900-1960s), which are listed in a recent bibliography of anopheline egg descriptions (Reinert 2010). An SEM is often required to visualize minor morphological differences that are characteristic of the eggs of closely related species, but because such facilities are expensive and often inaccessible, this method of identification is not common. Nonetheless, egg morphology represents a reliable method for accurately distinguishing *An. perplexens* and *An. smaragdinus* from morphologically similar native species (*An. punctipennis* and *An. quadrimaculatus s.s.*, respectively) (Linley *et al.* 1993; Linley and Kaiser 1994), and has potential to discover other previously unrecognized cryptic species. An SEM was

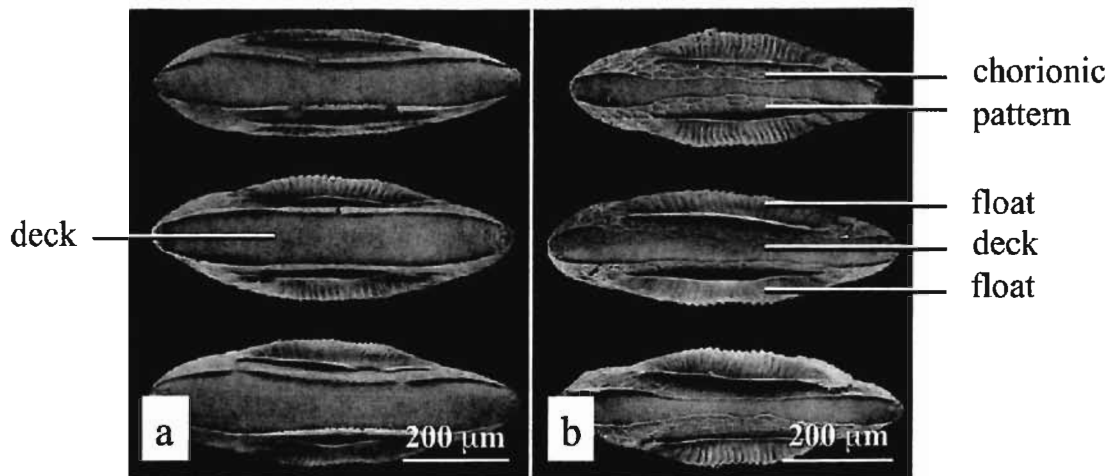

Figure 2.1. Eggs of *Anopheles punctipennis* and *Anopheles perplexens* (ventral view) (Figure 2 from Linley and Kaiser 1994). Deck is wide and slipper-shaped in *An. punctipennis*, with chorionic pattern not visible between deck and float (a). Deck is narrow in *An. perplexens*, with chorionic pattern of plastron cells visible between deck and float (b).

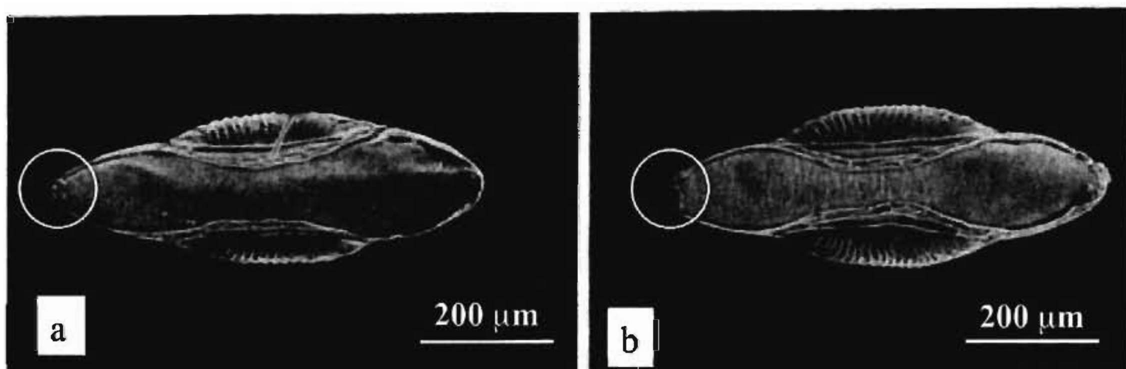

Figure 2.2. Eggs of *Anopheles quadrimaculatus s.s.* and *Anopheles smaragdinus* (ventral view) (Figure 18 from Reinert *et al.* 1997). The number of posterior basal tubercles (indicated by circles) is five in eggs of *An. smaragdinus* (a) and seven in *An. quadrimaculatus* (b).

accessible at Brock University and eggs from wild-caught females were examined for evidence of cryptic species in this study.

2.1.2. Larval Morphology

Larval morphology is often used to identify anopheline species and has been described in great detail, including the complete chaetotaxy (i.e., description of all setae) (White 1977; Wood *et al.* 1979). Setae are hair-like projections that arise from sockets in the body surface, or integument (Wood *et al.* 1979). Identification keys require fourth instar larvae because diagnostic characters are unreliable in immature larvae. Thus, it is often easier to allow early instars to mature than to attempt identification directly (Wood *et al.* 1979). Species-level identification of mosquito larvae is based on the arrangement and numbers of setal branches. The larval body is divided into three main segments, including the head, thorax, and abdomen (Figure 1.3), over which setae are arranged symmetrically in pairs.

Anopheles larvae differ from those of other mosquito genera that are present in Canada (except *Uranotaenia*) by the length of the respiratory siphon, which is greatly reduced in *Anopheles*, causing larvae to rest horizontal beneath the water's surface rather than hanging vertically from an elongated siphon (Figure 2.3). For setal nomenclature and detailed morphological descriptions of the *Anopheles* larvae in Canada, see Wood *et al.* (1979), and for descriptions of species previously known only from the USA, see Carpenter and LaCasse (1955). The types of setae commonly used in the identification of Canadian anophelines include: simple, branched, palmate, and plumose (Figure 2.4).

When identifying *An. earlei* larvae, additional accessory tergal plates (ATPs) (i.e., round, darkly-pigmented spots) were observed on abdominal segments III-VII, which were located below the single ATP present on the abdominal tergites of *all* anopheline larvae (Figure 2.5). Additional ATPs are the diagnostic character for identification of *An. freeborni* in both available identification keys for the mosquitoes of Canada and North America, north of Mexico (Wood *et al.* 1979; Darsie and Ward 2005). Additional ATPs are not mentioned or included in any of the couplets or illustrations of *An. earlei*

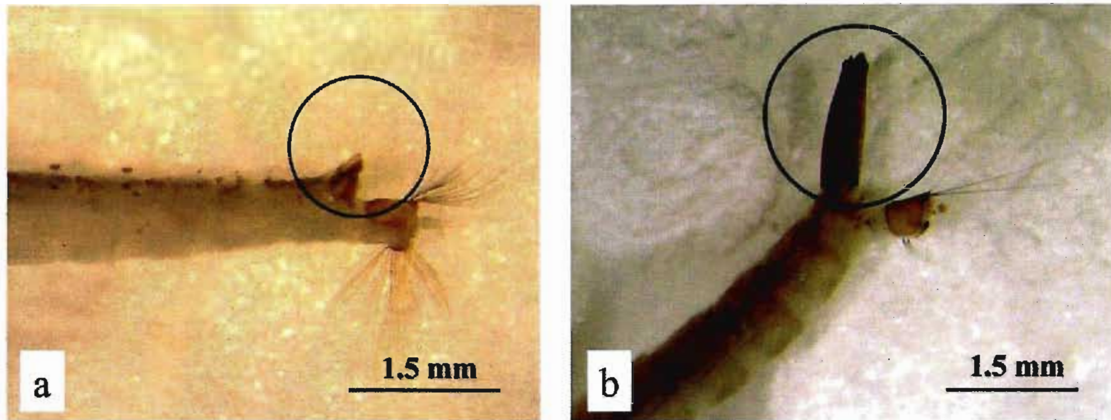

Figure 2.3. Respiratory siphon of *Anopheles* and *Aedes* larvae (lateral view). Circles indicate the reduced respiratory siphon of *Anopheles* larvae (a) and the elongated siphon of *Aedes* larvae (b).

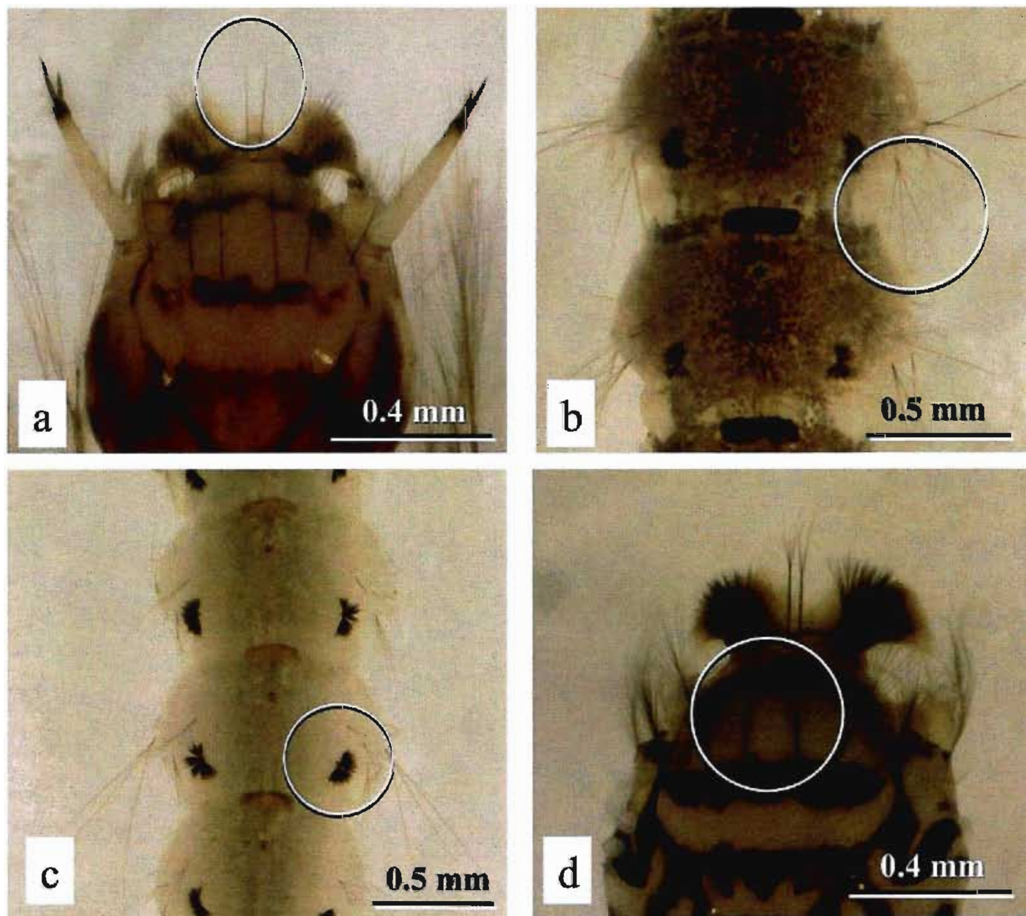

Figure 2.4. Common types of setae in *Anopheles* larvae (dorsal view), indicated by circles, including: a) simple, b) branched, c) palmate, and d) plumose.

specimens in either key. However, Hampton and Lawson (1967) clearly show 3 pairs of ATPs on the dorsal surface of abdominal segments IV-VI in their illustrations of both *An. earlei* and *An. freeborni* larvae. Illustrations of abdominal segment IV in Pratt (1952) indicate their occurrence in both species, and in *An. occidentalis* and *An. aztecus* as well. *An. earlei*, *An. freeborni*, *An. occidentalis*, and *An. aztecus* are all members of the *An. maculipennis* complex (White 1978), which includes about nine additional isomorphic species in the Palearctic region (Porter and Collins 1996). Since *An. earlei* is broadly distributed in the Nearctic region (Figure 1.9), the possibility of New World cryptic species must also be considered. Accordingly, morphological analyses included geographic comparisons of additional ATPs among *An. earlei* larvae collected from across Canada.

In addition, because cryptic species that are suspected to be in Canada (*An. occidentalis*, *An. perplexens* and *An. smaragdinus*) can sometimes be distinguished from native cryptic species (i.e., *An. earlei*, *An. punctipennis*, and *An. quadrimaculatus s.s.*, respectively) based on minor morphological differences in the larval stage, these characters were examined in larvae collected from various regions across their ranges. Detailed descriptions of these morphological characters are provided in section 2.1.4. Cryptic Species Potentially Occurring in Canada.

2.1.3. Adult Morphology

The morphology of adult mosquitoes has been thoroughly studied due to their importance as potential vectors of disease (see Chapter One). *Anopheles* adults differ from those of other mosquito genera in the structure of male genitalia and female palpi (White 1977; Wood *et al.* 1979). The thorax and abdomen of adult anophelines have numerous setae, but are almost completely devoid of scales and the palpi of *Anopheles* females are elongated (Figure 2.6). Although species-level identification of most male mosquitoes requires dissection and slide mounting of the internal portions of the genitalia, males of the anophelines present in Canada be identified based on the same external characters used for females (Wood *et al.* 1979). The characters used most often in the identification of *Anopheles* adults include presence or absence of dark-scaled spots

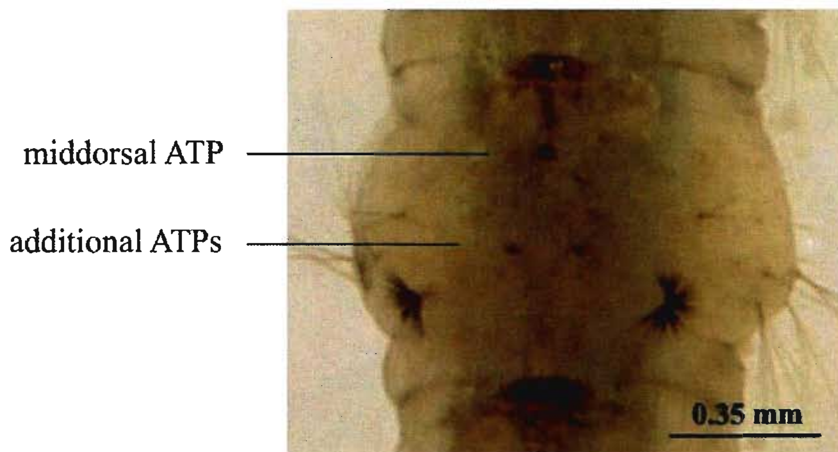

Figure 2.5. Additional accessory tergal plates (ATPs) in *An. freeborni* and *An. earlei* larvae (dorsal view). All *Anopheles* larvae have a single ATP located mid-dorsally on each tergite. All *An. freeborni* larvae, and some *An. earlei* larvae, have additional ATPs located below the single ATP on tergites III-VI.

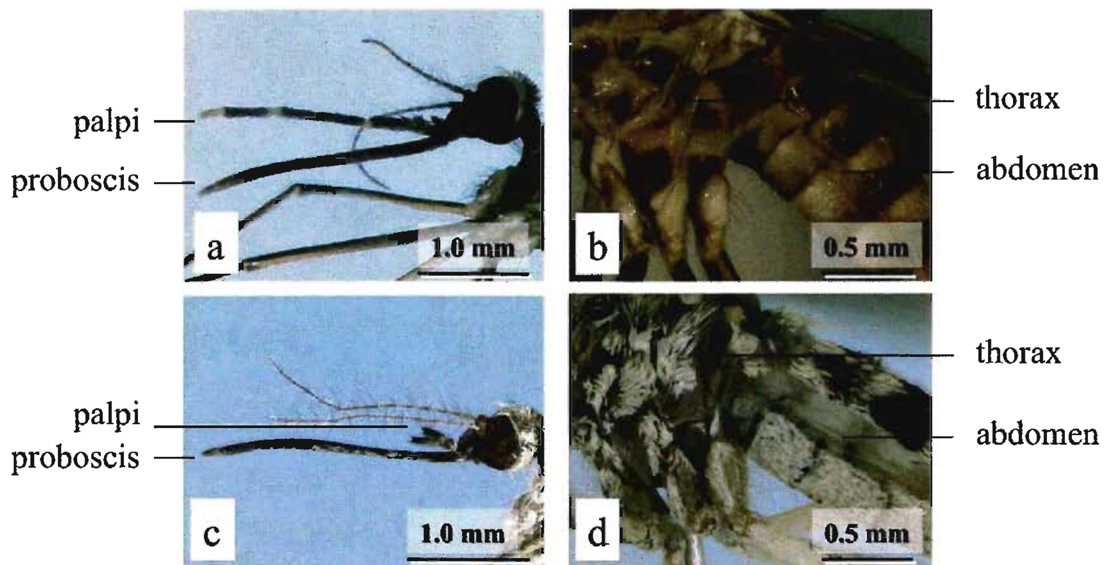

Figure 2.6. Morphology of *Anopheles* and *Aedes* adult females (lateral view). *Anopheles* females have elongated palpi (a), with thorax and abdomen without scales (b). Female mosquitoes of most other genera have reduced palpi (c), with scales covering thorax and abdomen (d).

on the wings (formed by aggregations of scales near bifurcations of wing veins), as well as scale patterns on the leg and palpus (entirely dark-coloured scaled or with pale-scaled bands).

As in the larval stage, the minor morphological differences used to distinguish cryptic species that potentially occur in Canada are described below, and were examined in adults to quantify the variation present.

2.1.4. Cryptic Species Potentially Occurring in Canada

In addition to the known species from Canada (*An. barberi, An. earlei, An. freeborni, An. punctipennis, An, quadrimaculatus s.l., An. walkeri*, and recently *An. crucians s.l.*), some anopheline species are suspected to occur in Canada. These include *An. occidentalis* in western Canada, and *An. perplexens* and *An. smaragdinus* in southern Ontario (see Chapter One for a description of these species and their potential to occur in Canada). These potentially occurring cryptic species can sometimes be distinguished from native species based on minor morphological characters, so detailed morphological analyses of larval and adult specimens were conducted to quantify the level of variation in diagnostic characters.

Authors of early studies involving *Anopheles* mosquitoes in northwestern North America mistakenly reported *An. occidentalis* from British Columbia (Rempel 1950) or throughout North America (Pratt 1952). However, detailed examination of the specimens in question by Wood *et al.* (1979) revealed that the central portion of wing vein R_2+R_3 was devoid of scales only on the upper side of the wing, as in *An. earlei*. Wood *et al.* (1979) hypothesize that, since the wings are transparent and scales on the lower side can be seen when viewed from above, early authors mistook them for scales on the dorsal surface of wing vein R_2+R_3, as in *An. occidentalis*. In Darsie and Ward (2005), the distinction between the two species is described as *An. earlei* with numerous erect scales on the dorsal surface of wing vein R_2+R_3 and decumbent (lying flat) scales on the ventral side of R_2+R_3 which are not visible from dorsal aspect, and, in *An. occidentalis,* wing vein R_2+R_3 is usually bare on dorsal surface with decumbent scales on ventral surface

that are visible from dorsal aspect. To date there are no confirmed records of *An. occidentalis* from Canada (Wood *et al.* 1979; Belton 1983).

In the larval stage, morphological differences between *An. occidentalis* and *An. earlei* are more obvious. The larvae of *An. occidentalis* are easily separated from those of *An. earlei* based on head setae 2-C, which are simple in *An. occidentalis* and forked in the distal half in *An. earlei* (Figure 2.7). However, using the key of Darsie and Ward (2005), *An. occidentalis* and *An. perplexens* larvae are distinguishable based only on collection location, with *An. occidentalis* present in the west and *An. perplexens* in the east. Both species have clypeal setae 2-C simple, with alveoli (cuticular pits from which setae originate) closer together than the diameter of one alveolus and setae 2-IV,V usually single.

Anopheles perplexens was suspected to be present in Canada based on morphological identification of adult females collected in Ontario during 2005-2009 field seasons, as well as through WNv mosquito surveillance at Brock University from 2001-2005. Pale-scaled wing spots are present in the adults of both species, but the size of the spot is *usually* reduced in *An. perplexens* (Figure 2.8). Darsie and Ward (2005) describe this difference as the subcostal pale spot (SCP) 0.5 or more the length of preapical dark spot (PAD) in *An. punctipennis*, and with SCP much reduced in *An. perplexens*, usually 0.33 or less the length of the PAD. This description does not take into account specimens with intermediate SCP lengths (e.g., SCP 0.4 the length of PAD). In a study of wing spot patterns in *An. punctipennis* and *An. perplexens*, Fritz *et al.* (1991) found that most specimens had SCP lengths of between 0.33 to 0.5 the length of the PAD. Accordingly, the identity of such specimens was questionable, and the character was considered unreliable for distinguishing the two species.

Larvae of *An. perplexens* and *An. punctipennis* can sometimes be distinguished by the number of branches of abdominal setae 2-IV and V, which are branched in *An. punctipennis* (usually 2-3) and usually single in *An. perplexens* (Figure 2.9); however, this character is known to be unreliable as *An. perplexens* larvae sometimes have setae 2-IV, V branched, as in *An. punctipennis* (Darsie and Ward 2005). Therefore, examination of larval setae 2-IV, V in *An. punctipennis* specimens was conducted and the number of branches determined to quantify variation among specimens collected from Canada.

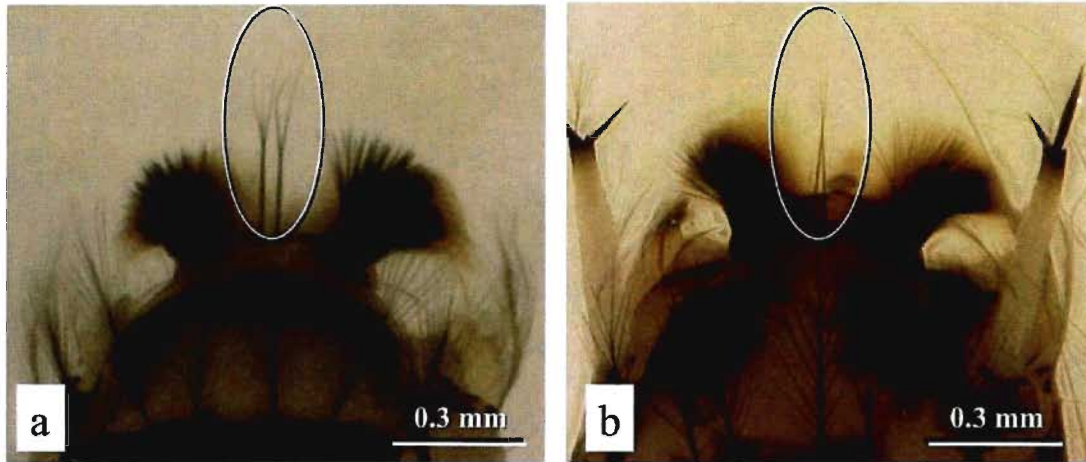

Figure 2.7. Larval head setae 2-C of *An. earlei* and *An. occidentalis (*dorsal view). Circles indicate setae 2-C, which are forked in the distal half in *An. earlei* (a), and simple in *An. occidentalis* (b). (Note: the image in (b) is of *An. punctipennis*, which have setae 2-C simple as well, to show how setae 2-C would look in *An. occidentalis*).

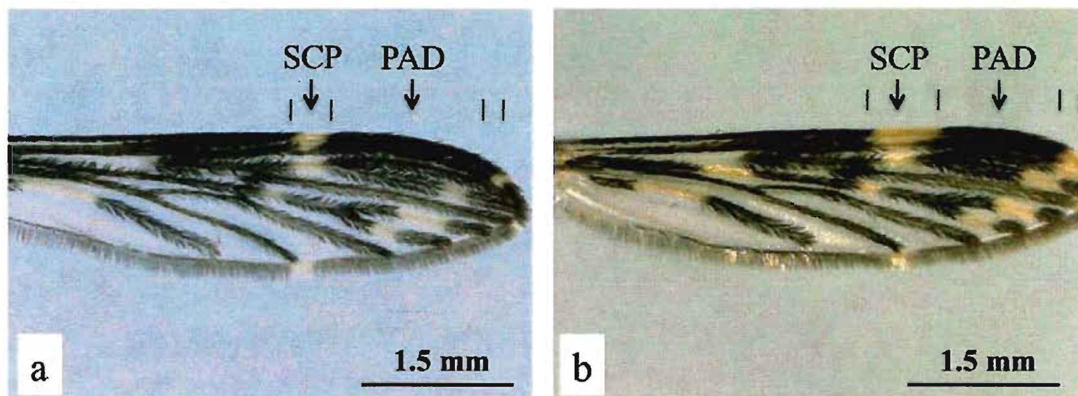

Figure 2.8. Wing morphology of *An. perplexens* and *An. punctipennis* (dorsal view). The subcostal pale spot (SCP) along the anterior margin of the wing (costa and adjacent veins) is *usually* greatly reduced in *An. perplexens*, i.e., 0.33 or less the length of the preapical dark spot (PAD) (a). The SCP is *usually* larger in *An. punctipennis*, i.e., 0.5 or more the length of PAD (b).

The last species warranting in-depth analysis is *An. quadrimaculatus s.l.,* now recognized as a complex of five sibling species in the southern USA: *An. quadrimaculatus s.s., An. smaragdinus, An. maverlius, An. diluvialus,* and *An. inundatus* (Reinert *et al.* 1997). *Anopheles quadrimaculatus s.s.* is the most broadly distributed member of the complex, extending north into southern Ontario and Quebec in Canada (Figure 1.12) (Levine *et al.* 2004). While most members of this complex have limited distributions in the southeastern US, *An. smaragdinus* has a broader range, predicted to extend as far north as southern New York and Pennsylvania, Ohio, and Indiana (Figure 1.16) (Levine *et al.* 2004), not far from the Canadian border.

Keys to the adult and larval members of the *An. quadrimaculatus* species complex are based on minor morphological differences, such as the number of setae arising from the thoracic scutal fossa and the combined number of branches of outer clypeal setae 3-C (Figure 2.10) (Reinert *et al.* 1997). *An. quadrimaculatus s.s.* adult females have 21 or more setae arising from the scutal fossa, while those of *An. smaragdinus* have 20 setae or less (Reinert *et al.* 1997). The combined number of branches of both head setae 2-C of *An. quadrimaculatus s.s.* is >64 (bunched distally), whereas those of *An. smaragdinus* total <63 (widely spaced distally) (Darsie and Ward 2005). Therefore, these and other distinguishing characters were examined in *An. quadrimaculatus s.l.* specimens collected from southern Ontario for evidence that *An. smaragdinus* occurs in Canada.

2.2. Methods

2.2.1. Mosquito Collections

Adult and larval mosquitoes were collected from widely separated locations across Canada to obtain specimens from as much of their range as possible. Collections were conducted in four representative regions including the West (British Columbia), Central (Ontario), North (Radisson, Quebec), and East (Newfoundland), and from four main and four incidental regions in Ontario including Windsor/Essex County, the Niagara Region, Manitoulin Island, and Ottawa, as well as Algonquin Provincial Park, the Bruce Peninsula, and Long Point Provincial Park (Figures 2.11, 2.12 and 2.13).

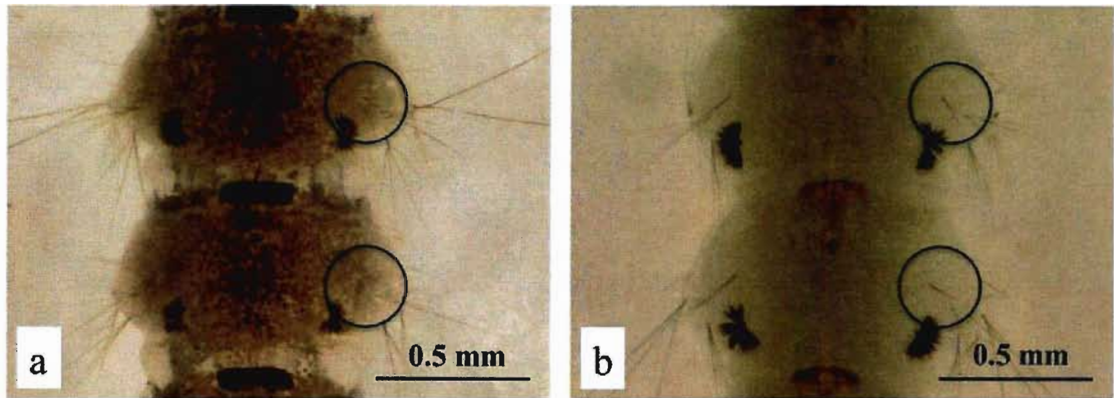

Figure 2.9. Larval abdominal setae 2-IV,V of *An. perplexens* and *An. punctipennis* (dorsal view). Circles indicate setae 2-IV,V with 2-3 branches in *An. punctipennis* (a) and are single in *An. perplexens* (b). (Note: the image in (b) is of *An. quadrimaculatus*, but is included to show what setae 2-IV,V would look like in *An. perplexens.*)

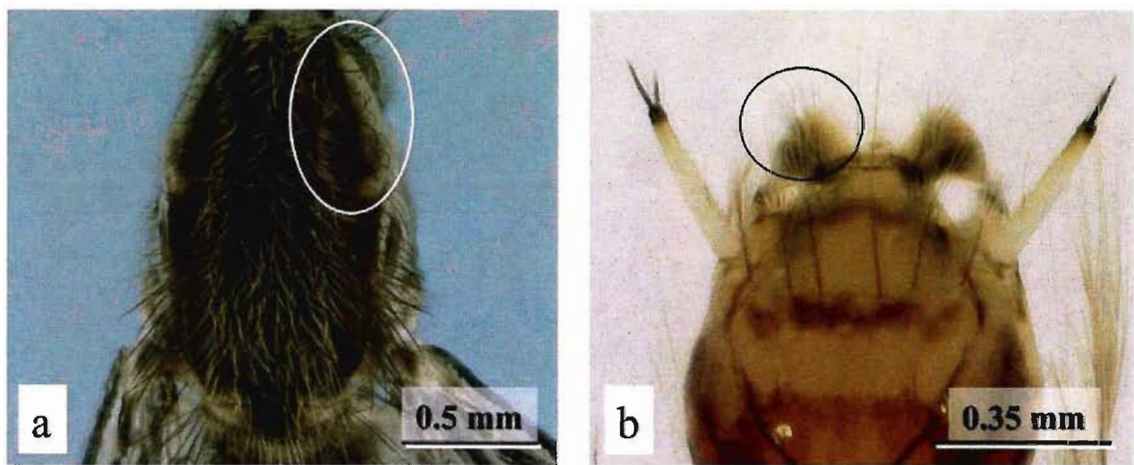

Figure 2.10. Characters used in discrimination of *An. quadrimaculatus s.s.* and *An. smaragdinus* (dorsal view). Adults are distinguishable based on the number of setae arising from the scutal fossa (a), and larvae based on the combined number of branches of setae 3-C, visible above and extending past the labral brushes (b).

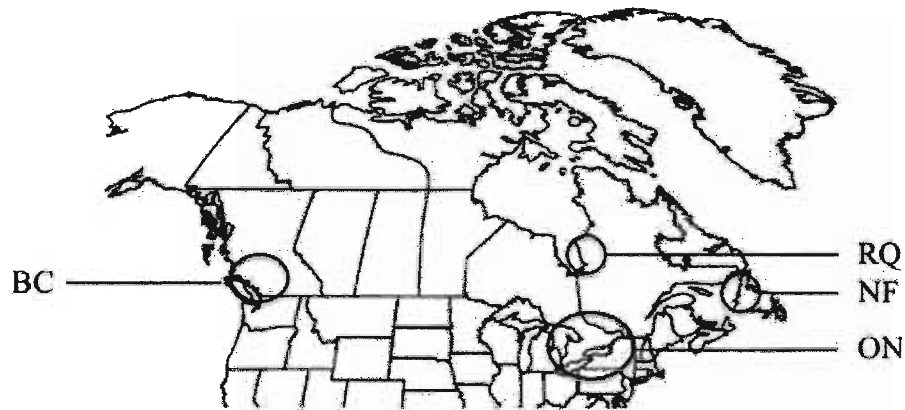

Figure 2.11. Four main *Anopheles* collection regions in Canada. The open circles indicate general collection locations. Site name and abbreviations listed in Appendix I.

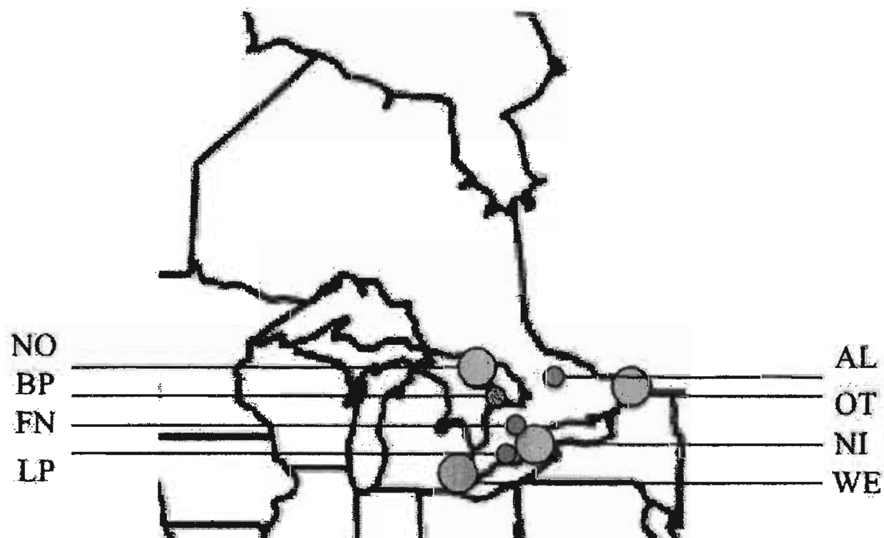

Figure 2.12. Four main and four incidental *Anopheles* collection locations in Ontario. Large, light grey circles represent regions from which multiple collections were made in two or more years. Small, light grey circles represent regions from which few collections were made in one year only. Site name and abbreviations listed in Appendix I.

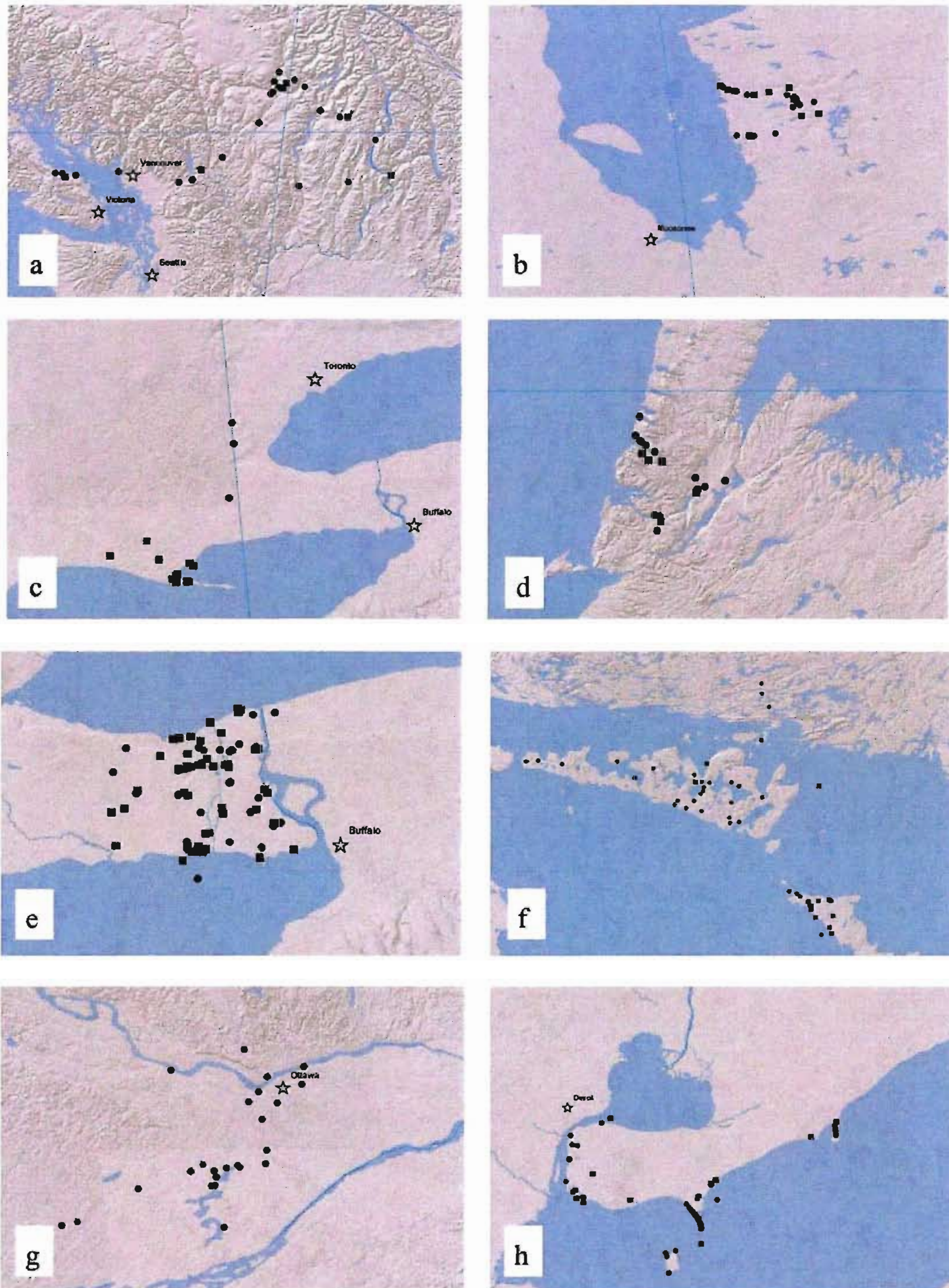

Figure 2.13. Adult trapping and larval dipping collection sites during 2005-2009 field seasons, with main collecting regions including: British Columbia (a), northern Quebec (b), Long Point (c), Newfoundland (d), Niagara (e), Manitoulin Island (f), Ottawa (g), and Windsor (h). Circles represent larval dipping collections and squares adult trap or landing aspiration collections. Site names and abbreviations listed in Appendix I.

for examination on a regional scale. Extra sampling effort was made in southern Ontario because almost all species present in Canada occur in that region and it is near known collection locations of suspected cryptic species *An. perplexens* and *An. smaragdinus*. Sampling region abbreviations are listed in Appendix I, and collection data are given in Appendix II.

Larvae were collected from the surface of standing water, in a wide variety of habitats (see Chapter Four), using standard larval dippers, a large white plastic tray, a very large pipette, and Whirlpak® sample bags (www.enasco.com). The dipper was skimmed across the water to collect larvae resting or feeding at the surface and the contents emptied into the plastic tray. Larvae were removed from the tray using the pipette, and placed into a Whirlpak® bag half-filled with water from the collection site plus small bits of vegetation and plant matter, which is sealed with air trapped inside the bag for transport to the lab. The bags were placed in a cooler containing cool water to absorb shock during transport and ensure survival to the lab.

Larvae were returned to the lab live in Whirlpak® bags and placed into mosquito breeders (Bioquip®, California, USA) with water from the collection site to allow development to fourth instar and adult stages (Figure 2.14). If insufficient food was collected with water from the collection site, larvae were fed small amounts of preferably plant-based fish food (e.g., Nutrafin® spirulina flakes), but sometimes fish-based fish food (e.g., Nutrafin® tropical fish flakes). Fish food was ground into a fine powder so that it would float on the water's surface for as long as possible, as larvae seemed to feed primarily at the water's surface. Samples were checked for predators, which were removed immediately if found. If water became foul, larvae were removed to dechlorinated tap water and fed fish food for the remainder of their larval development to the fourth instar stage. Emerging adult mosquitoes flew up into the adult mosquito chamber above the larval chamber (Fig. 2.14), and were provided with cotton dental wicks (Richmond Dental, North Carolina, USA) soaked in water and ~10% sucrose solutions, and allowed to survive for at least 24 hours to allow proper hardening of the integument.

To preserve larvae for morphological analysis, fourth instars were removed from breeders using small, disposable plastic pipettes and placed into hot water (60-70°C) for

approximately one minute prior to placing into glass scintillation vials filled with 80% ethanol for long term storage. Hot water treatment was used to prevent larvae from darkening over time, making morphological identification more difficult (Wood *et al.* 1979). However, this process is therefore not recommended because it destroys DNA that could otherwise be used for molecular analyses, and did not always prevent larvae from darkening. Larvae for molecular analyses were killed and preserved by placement directly into 95% ethanol. Adults reared from larvae were frozen at -20°C to kill and preserve them for long-term storage, a process that also sufficiently preserves the DNA for molecular studies.

Adult mosquitoes were collected during the 2005-2009 field seasons from locations throughout Canada (Figures 2.11, 2.12, and 2.13) using CDC light traps (Bioquip®, California, USA) and by landing aspirations. CDC light traps use ultraviolet light combined with dry ice (which releases a stream of carbon dioxide). This attracts mosquitoes, which are sucked into a retaining container by a fan (Figure 2.15). Traps were set up before dusk and collected after dawn the following day. Collection containers were kept cool and moist in coolers with freezer packs and paper towels to prevent desiccation and damage during transport, then returned live to the lab. Trap contents were placed into a freezer at -20°C to kill and preserve the insects, which were later sorted on a chill table (to keep frozen and preserve DNA) to separate *Anopheles* mosquitoes from other mosquitoes and insects. Specimens were returned to the freezer and stored at -20°C for storage until prepared for molecular analysis. Landing aspiration collections involved the use of hand-held aspirators to collect adult female mosquitoes from humans or indoor resting sites. Similar to adults obtained by other methods, those collected by landing aspirations were frozen at -20°C to kill and preserve them for subsequent analyses.

2.2.2. Species Identification

Larval and adult mosquitoes were identified using the keys of Wood *et al.* (1979) and Darsie and Ward (2005) under magnification with a Leica MS5 dissecting microscope. A character matrix was created (Table 2.1) and used for the identification of

Figure 2.14. Mosquito breeders used to rear *Anopheles* larvae and adults. Larvae and pupae develop in the water in the lower chamber and adults fly up through a funnel-shaped barrier to rest and feed in the upper chamber.

Figure 2.15. CDC light traps used to collect adult females mosquitoes.

Table 2.1. Character matrix for the identification of *Anopheles* larvae. Traits in bold represent diagnostic characters for species identification (Carpenter and LaCasse 1955; Wood *et al.* 1979; Darsie and Ward 2005). Species abbreviations: *barb* = *An. barberi*, *cruc* = *An. crucians s.l.*, *earl* = *An. earlei*, *free* = *An. freeborni*, *perp* = *An. perplexens*, *punc* = *An. punctipennis*, *quad* = *An. quadrimaculatus s.l.*, and *walk* = *An. walkeri*.

	head setae 5-C to 7-C	clypeal setae 2-C	thoracic seta 1-P	abdominal setae 0-IV and 0-V	"float hairs" 1-II	setae 2-IV and 2-V	additional tergal plates
barb	**simple**	simple, close	long, aciculate in outer half	minute	flattened into leaflets	branched	no
cruc	plumose	simple, close	short, aciculate near apex	**well developed, branched**	flattened into leaflets	more than three branches	no
earl	plumose	**forked,** close	weak, single, or branched in outer half	minute	**hair-like branches**	branched	sometimes
free	plumose	simple, close	weak, single, or branched in outer half	minute	flattened into leaflets	branched	**yes**
quad	plumose	simple, **far**	weak, single, or branched in outer half	minute	**partial to fully palmate**	usually single but may be double or triple	no
perp	plumose	simple, close	weak, single, or branched in outer half	minute	flattened into leaflets	**single**	no
punc	plumose	simple, close	weak, single, or branched in outer half	minute	flattened into leaflets	double or triple	no
walk	plumose	close, **aciculate in outer half**	**strong with 3-5 branches from base**	minute	partially palmate	**single**	no

questionable specimens since larval characters were sometimes difficult to ascertain.

A subset of *An. quadrimaculatus s.l.* larvae and adults were identified using the key to members of the *An. quadrimaculatus* species complex (Darsie and Ward 2005). All *An. earlei* larvae were examined for the presence of extra accessory tergal plates to see if there were any geographic patterns that might indicate the presence of a cryptic species. All *An. punctipennis* larvae and adults were examined for traits that usually distinguish them from those of *An. perplexens* (i.e., state of setae 2-IV,V and size of pale-coloured wing spot).

2.2.3. Scanning electron microscopy of eggs

Because the cryptic species suspected of being in Canada - *An. perplexens* and *An. smaragdinus* - can be reliably identified on the basis of egg morphology (Linley and Kaiser 1994; Linley *et al.* 1993), eggs obtained from adult females collected throughout the Niagara Region were examined using SEM. In studies of *An. perplexens* from Georgia and Florida, where that species is sympatric with *An. punctipennis,* the two species are known to inhabit different larval breeding grounds, with *An. perplexens* larvae occurring in the clear waters of limestone springs and *An. punctipennis* in other larval habitat types in the surrounding area (Bellamy 1956). Because many specimens collected in the Niagara region were identified as putative *An. perplexens* based on the size of subcostal pale spot, and because many limestone water habitats occur in Niagara, eggs from field-collected females were examined using SEM to determine whether this species actually occurs in southern Ontario.

Adult mosquitoes were collected throughout the Niagara region from near potential limestone larval habitats (such as old quarries and limestone springs) and returned live to the lab (methods as described above). Trap contents were then released into mosquito cages (30cm x 30cm x 30cm) and female *Anopheles* removed using a mouth aspirator to an empty mosquito cage. These females were later provided with a blood meal for egg development. In 2008, guinea pigs were restrained in wire cages using strips of Velcro and placed into mosquito cages for 5-20 minutes. The use of guinea pigs for provision of blood meals to mosquitoes was approved by the Brock

University Animal Care and Use Committee (Animal Use Project Proposal #08-02-02). However, because the guinea pigs appeared to undergo significant stress during the blood-feeding procedure, and since many had already begun to feed upon my arms in the field before they could be aspirated, wild-caught mosquitoes were allowed to feed on my arm during the 2009 field season. This is standard practice in many mosquito studies and WNv activity was monitored throughout the season to minimize potential health risk.

Blood fed females were isolated into separate oviposition chambers, made using a mosquito breeder with the plastic cone barrier between upper and lower chambers removed to allow females access to the entire chamber. Each chamber contained a small white plastic dish containing one cotton dental wick soaked in water and another soaked with a 10% sucrose-solution (water and food source), as well as a small black plastic dish containing water (dechlorinated tap water or water from larval habitat) as an oviposition substrate. Egg batches (~10-100 eggs) were left for at least 24 hours for the cuticle to harden properly. Then, ~5-20 eggs from each batch were removed from the water's surface for SEM analysis using a utensil made by taping a few paintbrush bristles to the end of a small wooden applicator stick.

To prepare eggs for scanning electron microscopy, two different methods were employed. The first, in 2008, was based on methods described by Linley and colleagues for the description of *Anopheles* eggs (Linley *et al.* 1993; Linley and Kaiser 1994), which involved fixation of eggs in Bouin's solution, then dehydration using increasing concentrations of ethanol (75%, 85%, 95%, and 100%), followed by critical point drying. Because a critical point dryer was not available at Brock University for this final step, a chemical treatment involving hexamethyldisilazane (HMDS) was used instead (Brown 1993). The eggs were then carefully oriented onto a carbon adhesive tab placed onto an Aluminum SEM stub. A conductive coating of gold/palladium was applied using a Polaron SC500 Sputter Coater, and the stubs then placed into the SEM by the technician. Using a secondary electron scintillation detector and 15kV accelerating voltage, images were processed using ORION Digital Image grabbing software.

Although the HMDS method has been used successfully to preserve small insects for SEM (Brown 1993), eggs became shriveled and/or brittle, which rendered them unsuitable for analysis using SEM. Therefore, in an attempt to obtain scanning electron

micrographs of eggs of sufficiently high quality, I tried using "fresh" eggs. Eggs were removed from the oviposition dish 24 hours after oviposition, placed directly onto black adhesive on an SEM stub, and then coated with gold/palladium and digital images were taken. This method, which resulted in significantly higher quality images that were sufficient for morphological analysis, was used thereafter.

2.2.4. Isofemale Progeny Broods (IPBs)

During the course of obtaining eggs for examination using the SEM, all eggs from one female were isolated and, after some were removed for SEM analysis, the rest were allowed to hatch and reared through the larval, pupal, and adult stages. This procedure resulted in ~20-200 siblings in what are known as isofemale progeny broods (IPBs), which allow for the comparison of egg, larval, and adult morphology. Specimens reared from *An. punctipennis* IPBs, including those identified as *An. perplexens*, were also examined to quantify variation in both the larval and adult characters used to identify these species (as described in Darsie and Ward 2005).

2.3. Results and Discussion

All species previously known from Canada were collected during the course of study, except *An. crucians s.l.*, which was recently introduced to southwestern Ontario and known from only a few specimens (Thielman and Hunter 2007). Considerable effort was made to recollect *An. crucians s.l.* near known collection sites in the Windsor region, but without success. Details about the number of specimens of each species collected and the geographic regions from where they were collected are provided in Appendix II. Analysis of ecological data associated with larval and adult collections is provided in Chapter Four. For consistency, the following results and discussion of species identifications and morphological analyses are organized alphabetically by species, similar to the approach used in the molecular (Chapter Three) and ecological (Chapter Four) analyses.

2.3.1. Anopheles barberi

Both the larvae and adults of *An. barberi* are morphologically very distinct from the other Anopheles species in Canada. In general, the larvae are smaller and have darker pigmentation than other anophelines, with the posterior end of the abdomen and the entire head, including antennae, very darkly pigmented (Figure 2.16). *Anopheles barberi* larvae are easily distinguished from those of other Canadian anophelines by the form of the head setae 5-C to 7-C, which are short and simple in *An. barberi*, but longer and plumose in other anophelines (Figure 2.16). Furthermore, abdominal lateral setae 6-IV to 6-VI are plumose in *An. barberi*, but are branched from the base in other anopheline larvae (Wood *et al.* 1979).

Adults of *An. barberi* are also easily distinguished from those of the other anophelines present in Canada. In general, they are smaller than other anophelines, and are the only species without a pattern of spots on their wings formed by aggregations of dark-coloured scales (Figure 2.17). In addition, scutal setae are dark, long, and erect in *An. barberi*, but are short, curved, and pale in other anopheline adults (Wood *et al.* 1979). These distinctive morphological traits, combined with the equally specific larval habitat of *An. barberi* (i.e., tree holes), make this species easy to identify.

2.3.2. Anopheles crucians s.l.

Although no specimens of *An. crucians s.l.* were collected during the 2005-2009 field seasons, a description of their morphology is included for completeness. Members of the *An. crucians* species complex are assigned to the Punctipennis Group (Figure 1.6), and although *An. crucians s.l.* shares similar morphologies with other members of this group (i.e., *An. punctipennis* and *An. perplexens*), they can be distinguished in both the larval and adult stages.

Larvae of *An. crucians s.l.* share several diagnostic characters with *An. punctipennis* and *An. perplexens*, but are distinguishable from these and other anophelines based on abdominal setae 0-IV and 0-V (Table 2.2), which are well developed with multiple branches in *An. crucians s.l.*, but minute in other

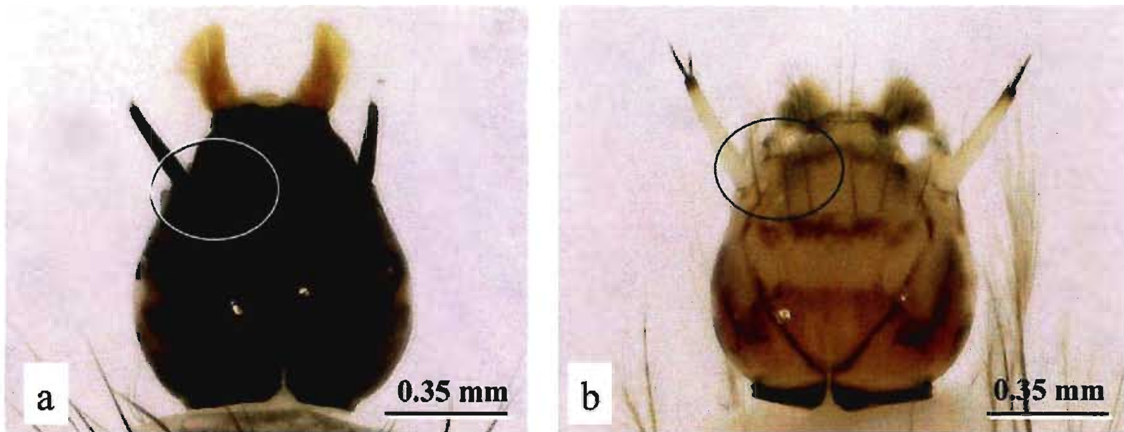

Figure 2.16. Head setae 5C-7C of *An. barberi* and *An. quadrimaculatus s.l.* larvae (dorsal view). Circles indicate head setae 5C-7C, which are short, simple, and gold-coloured (like labral brushes) in *An. barberi*, making them very difficult to see (a). Setae 5C-7C are larger, plumose, and dark-coloured in other anophelines (e.g., *An. quadrimaculatus*) (b).

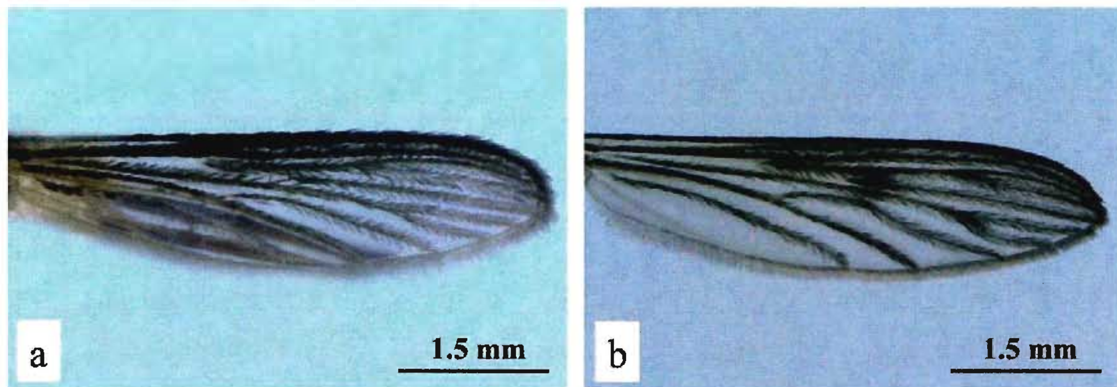

Figure 2.17. Wings of adult *An. barberi* and *An. walkeri* (dorsal view). *An. barberi* adults have dark-coloured wing scales distributed evenly along all wing veins (a), while those of other anophelines have wing scales aggregated at multiple vein bifurcations, forming a pattern dark spots on the wing, as in *An. walkeri* (b).

Canadian species (Figure 2.18a) (Darsie and Ward 2005). Like other members of the Punctipennis Group, adults of *An. crucians s.l.* also have pale-coloured scales on their wings. However, the pale-coloured wing spot does not include the anterior wing vein (Figure 2.18b), as in *An. punctipennis* and *An. perplexens* (Darsie and Ward 2005). *Anopheles crucians s.l.* adults also have pale-coloured scales at the distal ends of each palpomere, which results in palpi having pale-scaled bands along their length (Darsie and Ward 2005). The only other anopheline in Canada with pale-banded palpi is *An. walkeri*, which, unlike *An. crucians s.l.*, has completely dark-scaled wings (Figure 2.17b).

2.3.3. Anopheles earlei

Anopheles earlei is the most widely distributed anopheline in Canada, occurring in almost every province. It is also easily distinguished from the other Canadian anopheline species in both the larval and adult stages. Larvae of *An. earlei* have clypeal setae 2-C forked in the distal half, a trait that distinguishes them from larvae of other anophelines (Figure 2.19a). *Anopheles earlei* adults are distinct with a patch of copper-coloured fringe scales at the apex of their otherwise dark-scaled, spotted wings (Figure 2.19b). Adults of closely related *An. occidentalis* (currently known only from the USA) also have a patch of copper wing fringe scales, but they can be distinguished from *An. earlei* based on the dorsal surface of wing vein R_2+R_3 (described above) (Darsie and Ward 2005). *Anopheles occidentalis* was previously known from California and Oregon (Figure 1.14) (Darsie and Ward 2005), and has recently been found further north in the state of Washington (Sames *et al.* 2007). Therefore, all *An. earlei* adults collected from BC were re-examined for this trait.

Of the five specimens that were available for examination, three had erect scales visible on the dorsal surface of wing vein R_2+R_3 as in *An. earlei*, one had that part of the wing curled and vein R_2+R_3 not visible, and one appeared to have decumbent instead of erect scales on the dorsal surface of R_2+R_3. However, this particular specimen was used in molecular analyses, which confirmed its initial identification as *An. earlei*. So, while there are no confirmed records of *An. occidentalis* from BC, this trait should be examined

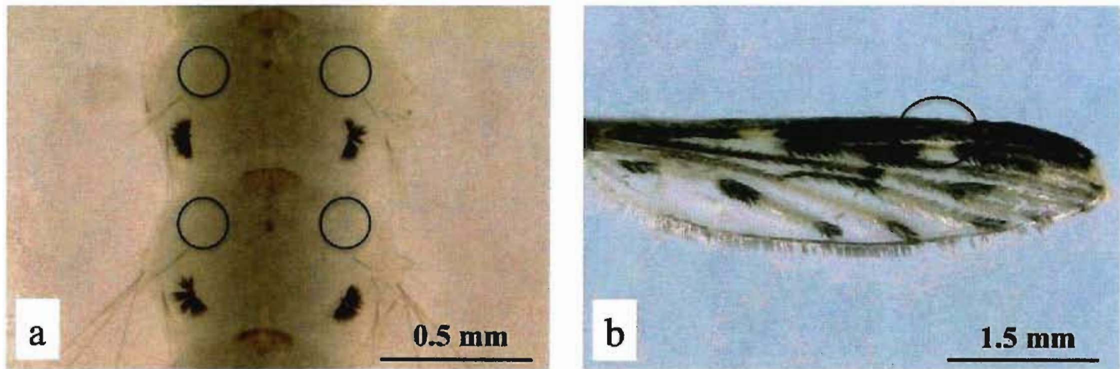

Figure 2.18. Distinguishing characters of *An. crucians s.l.* larvae and adults. Open circles indicate location of setae 0-IV,V in (a), which are well developed with multiple branches in *An. crucians s.l.*, but are minute, in other anophelines. Adults of *An. crucians s.l.* have pale-coloured wing spots, indicated in (b) by an open circle, but pale scales do not extend to the anterior costal and subcostal veins.

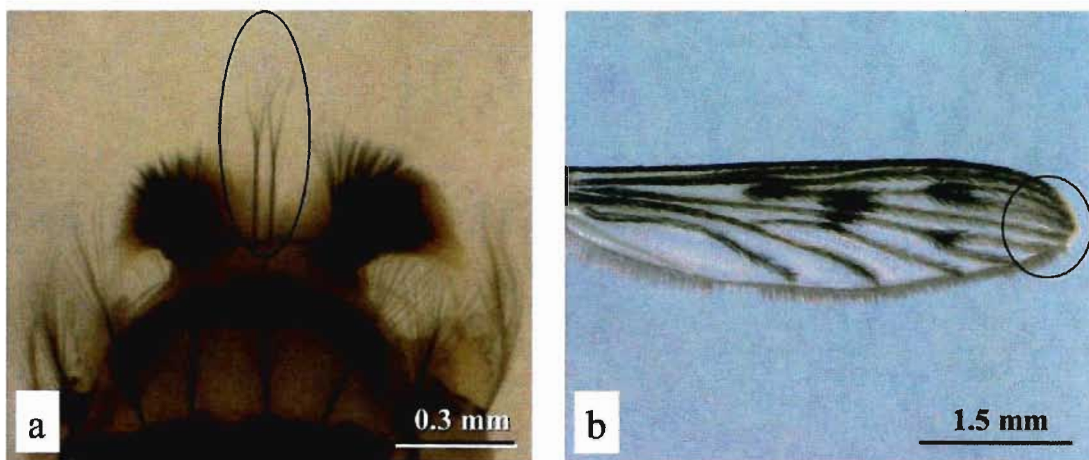

Figure 2.19. Distinguishing characters of *An. earlei* larvae and adults. Larvae have head setae 2-C forked in the outer half (a). Adults have copper-coloured fringe scales at the apex of the wing (b).

in *An. earlei*-type specimens collected from the province to determine whether the range of *An. occidentalis* has expanded northward into British Columbia.

Anopheles earlei larvae were examined for the presence of additional accessory tergal plates (ATPs) on the dorsal surface of abdominal tergites. Unlike *An. freeborni*, which have 8 additional ATPs arranged in symmetrical pairs on tergites III-VI, the number of additional ATPs varied among *An. earlei* specimens from 1-10 on tergites III-VII. A total of 136 *An. earlei* larvae were identified and the number of additional ATPs recorded for each specimen. Specimens with and without additional ATPs were present in most regions, except Niagara (all larvae without ATPs) and Newfoundland (all larvae with ATPs), but samples sizes were very low in both regions (n=3 and n=4, respectively) (Table 2.2). Of the four other regions (AL, BC, NO, and OT), additional ATPs were present in 50.0-76.5% of specimens from each region (Table 2.2).

Overall, additional ATPs were present in 65.4% of larvae examined, but the number of ATPs per specimen was highly variable (Table 2.2). Unlike *An. freeborni*, the number and arrangement of ATPs in *An. earlei* were not consistent; they could be present on any of tergites III to VII, were not necessarily located on adjacent tergites, and were not always arranged in symmetrical pairs (Figure 2.20). For example, a specimen with 6 ATPs may have 3 pairs of ATPs on tergites IV-VI, 3 pairs on tergites IV, V, and VII, or with 2 ATPs on tergite IV, 1 on tergite V, 1 on tergite VI, and 2 on tergite VII, etc. However, ATPs were only found on tergites III-VII, and none on tergites II or I. This is the first report of such non-symmetrical organization of ATPs in *An. earlei,* or any other anopheline species.

There were no obvious patterns in the presence or absence of additional ATPs based on region of collection (i.e., specimens with and without ATPs were found in all regions with samples sizes greater than seven) or collection location (i.e., from the same larval dipping or adult trap collection). Therefore, this particular morphological trait does not appear to be indicative of a cryptic species within *An. earlei* in Canada.

Table 2.2. Number of additional accessory tergal plates present in *An. earlei* larvae. Region codes are listed in Appendix I.

	No. specimens examined	0	1-2	3-4	5-6	7-8	9-10	% ATPs absent	% ATPs present
AL	17	4	1	1	5	6	0	23.5	76.5
BC	8	4	0	2	0	1	1	50.0	50.0
NF	4	0	1	1	0	2	0	0.0	100.0
NI	3	3	0	0	0	0	0	100.0	0.0
NO	88	32	14	8	10	13	11	36.4	63.6
OT	16	4	3	1	1	5	2	25.0	75.0
Total	136	47	19	13	16	27	14		

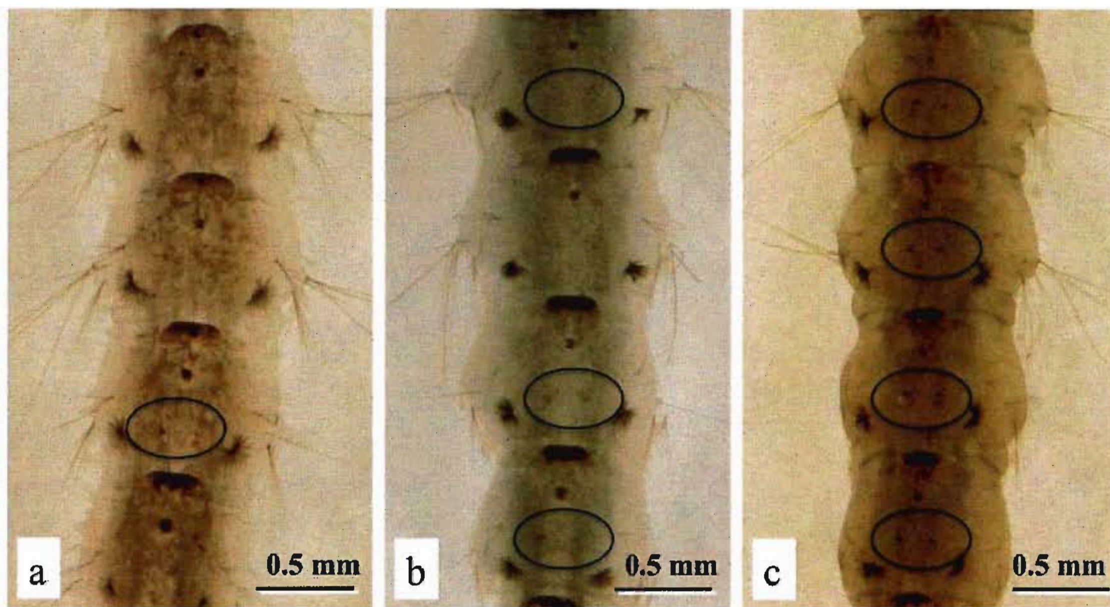

Figure 2.20. Possible arrangements of additional ATPs in *An. earlei* larvae. Examples of variable numbers of ATPs present in *An. earlei* larvae, indicated by open circles, including: one (a), six (b), and eight (c). ATPs were not always in organized in symmetrical pairs, sometimes with only one unsymmetrical ATP present per tergite (a).

2.3.4. Anopheles freeborni

The range of *An. freeborni* in Canada is relatively small, restricted to the southern interior of British Columbia (Figure 1.10), and morphological examination of larval and adult specimens collected did not reveal the presence of cryptic species. *Anopheles freeborni* larvae are easily distinguished from those of the other anopheline species present in Canada. They have two additional ATPs on segments IV-VII, which are considered diagnostic for this species in both available identification keys (Figure 2.21a) (Wood *et al.* 1979; Darsie and Ward 2005). Most specimens examined had the same spots on tergite III as well, for a total of 10 extra accessory tergal plates. However, as mentioned in the section above, additional ATPs are also present in *An. earlei*, which is sympatric with *An. freeborni* in BC, though the two species differ in head setae 2-C, which is forked in the distal half in *An. earlei* (Figure 2.19a) and simple in *An. freeborni* (Figure 2.21b) (Wood *et al.* 1979; Darsie and Ward 2005).

Anopheles freeborni adults are similar to those of *An. quadrimaculatus s.l.*, with wings and palpi entirely dark-scaled, and aggregations of dark wing scales forming a pattern of spots (Figures 2.22a). Darsie and Ward (2005) include a minor morphological difference between these two species based on the shape of scales located at the base of the cubital wing vein, which are linear and truncate apically in *An. freeborni* (Figure 2.22b), but are more oval-shaped and rounded apically in *An. quadrimaculatus s.l.* (Figure 2.28b). However, these two species are easily distinguished in Canada based on locality information as *An. freeborni* occurs in the west (Figure 1.10), whereas *An. quadrimaculatus s.l.* occurs in the east (Figure 1.12).

2.3.5. Anopheles punctipennis

Anopheles punctipennis is present in the southern parts of most provinces (Figure 1.11), with the larvae more easily confused with those of other anophelines from Canada compared to the adults. The larval identification key of Wood *et al.* (1979) requires the use of mounted specimens and a compound microscope to visualize the difference in

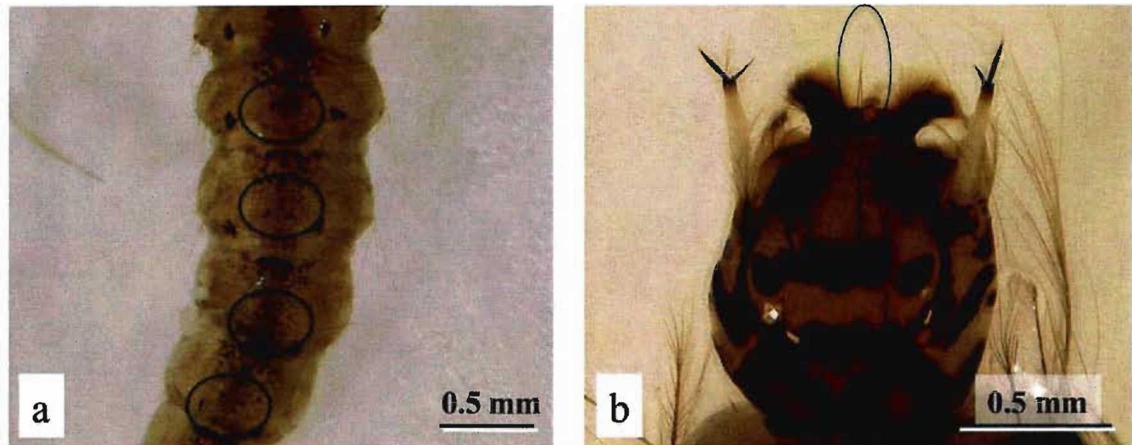

Figure 2.21. Distinguishing characteristics of *An. freeborni* larvae. Circles indicate Abdominal tergites IV-VII have additional ATPs (a). Clypeal setae 2-C are simple (b).

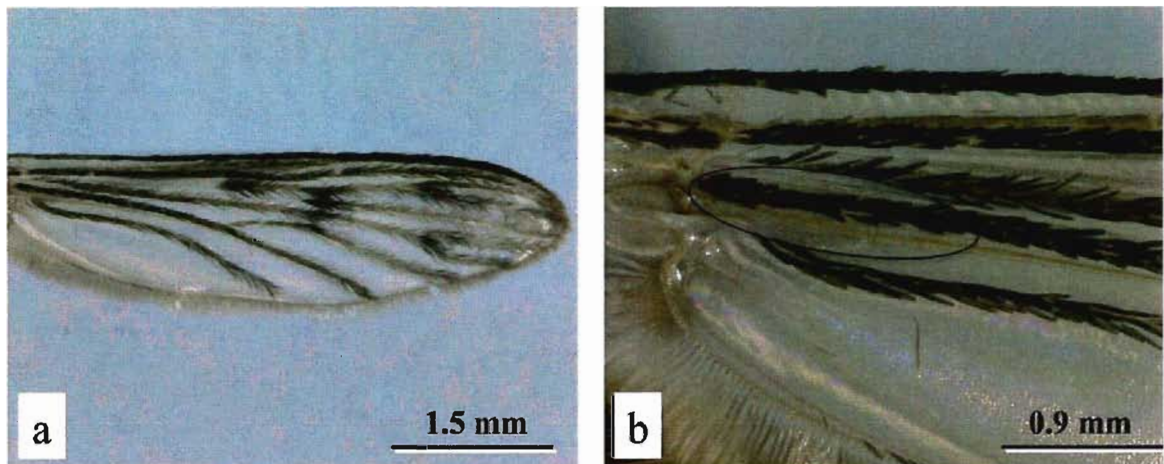

Figure 2.22. Distinguishing characteristics of *An. freeborni* adults. Legs, palpi, and wings are entirely dark-scaled, wing with aggregations of scales at vein bifurcations, forming pattern of spots (a). Wings are similar to those of *An. quadrimaculatus s.l.*, but can be distinguished by basal scales of the cubital vein, which are linear and truncate apically in *An. freeborni* (b), and more oval-shaped and rounded apically in *An. quadrimaculatus s.l.* (Figure 2.28b).

setae 2-C between *An. punctipennis* and *An. walkeri*, which are simple in *An. punctipennis* and aciculate near apex in *An. walkeri* (Figure 2.23). However, thoracic seta 1-P is also distinctive in *An. walkeri* larvae (Darsie and Ward 2005), providing a reliable character that can be used to distinguish these two species without the aid of higher magnification (Figure 2.24). Therefore, this character has been incorporated into the character matrix designed to aid in the identification of questionable larval specimens (Table 2.1).

Anopheles punctipennis adults are easily distinguished from most of the remaining *Anopheles* species present in Canada by the presence of pale scales on the wing that form a pattern of spots. Since the discovery of *An. crucians s.l.* in southern Ontario (Thielman and Hunter 2007), they are no longer the only species in Canada with pale-coloured wing scales. However, the main pale-coloured spot includes the costal vein in *An. perplexens* and *An. punctipennis* (Figure 2.8), but not in *An. crucians s.l.* (Figure 2.18b). Furthermore, identification of this species is complicated by the presence of *An. perplexens*-type specimens in southern Ontario. While the character used to discriminate *An. perplexens* and *An. punctipennis* (i.e., SCP size; Figure 2.8) is not completely reliable (Bellamy 1956; Fritz *et al.* 1991), variation within this trait was quantified and analysed to determine if geographic patterns were present.

Darsie and Ward (2005) describe the difference between the two species as the SCP 0.50 or more the length of PAD in *An. punctipennis*, and 0.33 or less the length of PAD in *An. perplexens*, which does not take into account intermediates, i.e., those with SCPs 0.34 to 0.49 the length of PADs. For the purpose of this study, adult anophelines were identified as *An. punctipennis* if the SCP was *greater than 0.33* the length of the PAD, and as *An. perplexens* if SCP was *less than 0.33* the length of the PAD.

In total, 135 of 1478 adults (9.1%) reared from larval dipping collections were identified as *An. perplexens* (Table 2.3). Sample sizes were low in four of the incidental collection regions (AL, BP, FN, and HL) and are therefore excluded from the following discussion. The relative proportion of specimens identified as *An. perplexens* and *An. punctipennis* varied depending on the region of collection. The highest proportion of adults identified as *An. perplexens* was found in OT (14.7%) and the lowest in BC and WE (0%) (Table 2.3). In their type locality, *An. perplexens* larvae are specific to alvars,

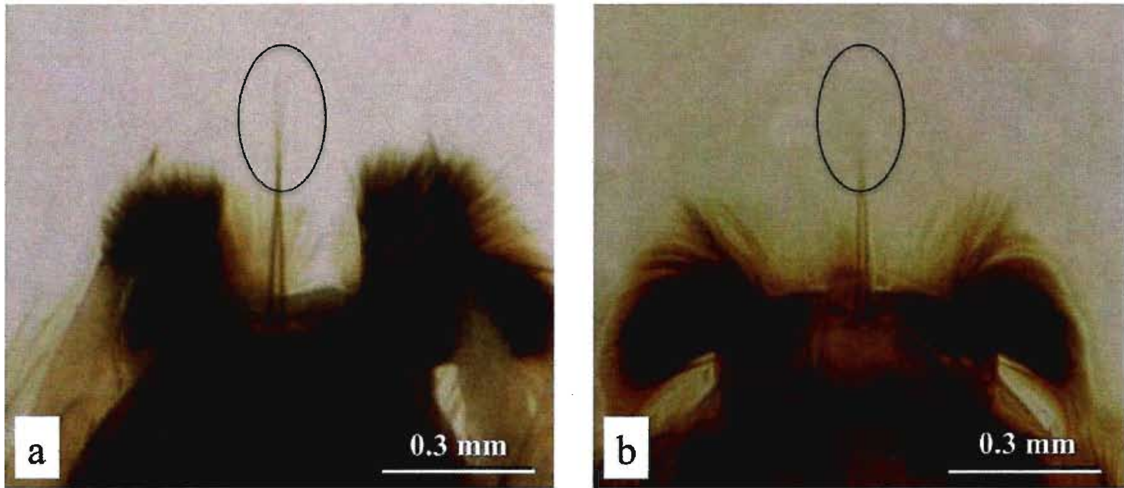

Figure 2.23. Larval head setae 2-C of *An. punctipennis* and *An. walkeri* (dorsal view). Circles indicate setae 2-C, which originate close together in both *An. walkeri* and *An. punctipennis* (closer than the diameter of one alveolus), but the apices of 2-C are aciculate in *An. walkeri* (a) and simple in *An. punctipennis* (b), a character not easily seen with a dissecting microscope.

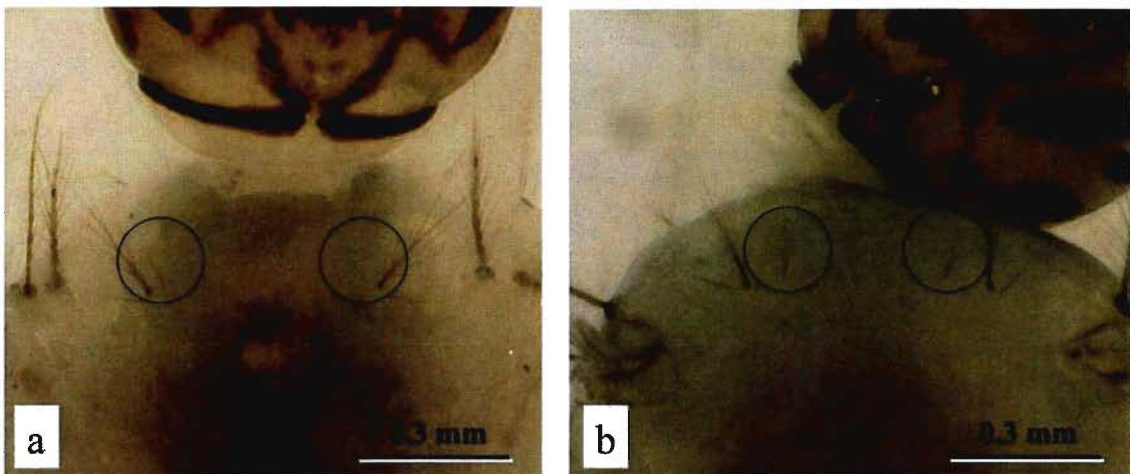

Figure 2.24. Larval thoracic setae 1-P of *An. punctipennis* and *An. walkeri* (dorsal view). Circles indicate setae 1-P, which are single in *An. punctipennis* (a), and well developed with 3-5 strong branches from base in *An. walkeri* (b).

bodies of water that form by seepage up through limestone (Bellamy 1956). Known alvars are located throughout NO and OT, and a number of alvars and alvar-type habitats were located in NI during the current study that were not recognized by the International Alvar Conservation Initiative (Reschke *et al.* 1999). In the five main regions in which *An. punctipennis* was found, *An. perplexens*-type adults were collected mainly from the regions where alvar habitats are known (NI, NO, and OT) (Reschke *et al.* 1999), which suggested that *An. perplexens* may be present in these areas (see Chapter Four).

In addition to adults reared from field-collected larvae, larvae and adults were reared from eggs obtained from females collected in the Niagara region for egg morphology studies. Larvae were identified as *An. punctipennis* if all setae 2-IV,V had two or more branches, and *An. perplexens* if even one of setae 2-IV,V was single. Of 282 larvae, 57 (20.2%) were identified as *An. perplexens*, as well as 89/509 (17.5%) of the adults (Table 2.4). A total of 791 larvae and adults were obtained from the eggs of 26 females, 25 of which were identified as *An. punctipennis* and one as *An. perplexens* (Table 2.5). Four of the eggs batches (15.4%) produced only *An. punctipennis* offspring, most egg batches (84.6%) produced offspring identified as both species, and none produced only *An. perplexens*. In addition, a wide range of SCP ratios were observed within single IPBs that produced both types of offspring (Figure 2.25). These data suggest that the variation observed in this trait likely represents intraspecific morphological variation within *An. punctipennis*, and cannot confirm the presence of cryptic species *An. perplexens* in the Niagara region. However, due to the low number of IPBs obtained from *An. perplexens* females, results of morphological analyses are inconclusive.

The large number of larvae and adults from regions where limestone larval habitats are present that were identified as *An. perplexens* based on the morphological characters suggested the potential presence of this species in southern Ontario (see Chapter Four). However, because both larval and adult morphology are known to be unreliable in the identification of this cryptic species, analyses of egg morphology (see below) and molecular data (see Chapter Three) were conducted to confirm the presence or absence of *An. perplexens* in southern Ontario.

Table 2.3. Relative proportion of specimens reared from larval dipping collections identified as *An. punctipennis* and *An. perplexens* using the key of Darsie and Ward (2005).

Region	Total	*An. punctipennis*		*An. perplexens*	
		n	%	n	%
AL	28	24	85.7	4	14.3
BC	141	141	100.0	0	0.0
BP	3	3	100.0	0	0.0
FN	5	4	80.0	1	20.0
HL	32	24	75.0	8	25.0
NI	657	566	86.1	91	13.9
NO	445	430	96.6	15	3.4
OT	109	93	85.3	16	14.7
WE	58	58	100.0	0	0.0
Total	1478	1343	90.9	135	9.1

Table 2.4. Relative proportions of larvae and adults reared from isofemale progeny broods that were identified as *An. perplexens* and *An. punctipennis*. Identifications of maternal females listed in Table 2.6.

	Total	*An. punctipennis*		*An. perplexens*	
		n	%	n	%
Larvae	282	225	79.8	57	20.2
Adults	509	420	82.5	89	17.5
Total	791	645	81.5	146	18.5

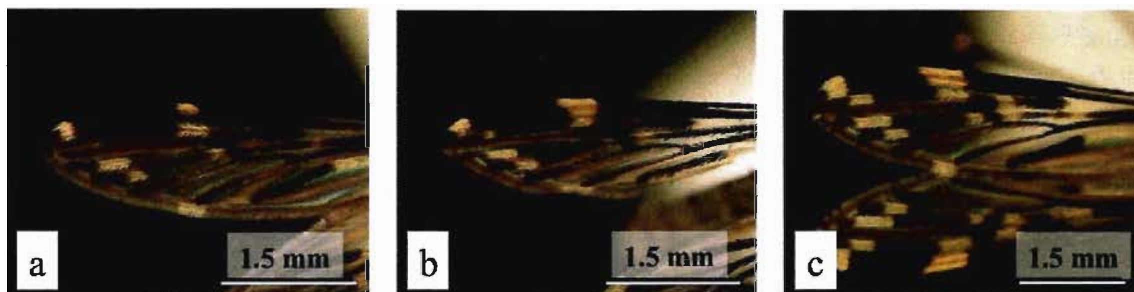

Figure 2.25. Wing pattern variability among three *An. punctipennis* siblings. Wide ranges in SCP lengths relative to the PADs were present among adult females reared from same the IPB, for example: 0.28 (a), 0.40 (b), and 0.67 (c).

Table 2.5. Identifications of *An. punctipennis* and *An. perplexens*-type females that produced IPBs, including SCP length and egg identifications (* indicates eggs with plastron visible between deck and float, but deck not as narrow as in *An. perplexens*).

Code	Parental Female	SCP length	Egg ID
NI203-3	*An. punctipennis*	1.00	*An. punctipennis*
NI216-3	*An. punctipennis*	1.00	*An. punctipennis*
NI216-12	*An. punctipennis*	0.80	*An. punctipennis*
NI216-10	*An. punctipennis*	0.80	*An. punctipennis*
NI216-8	*An. punctipennis*	0.80	*An. punctipennis*
NI109-1	*An. punctipennis*	0.80	*An. punctipennis*
NI160-3	*An. punctipennis*	0.80	*An. punctipennis*
NI203-5	*An. punctipennis*	0.67	*An. punctipennis*
NI203-7	*An. punctipennis*	0.67	*An. punctipennis*
NI206-18	*An. punctipennis*	0.67	*An. punctipennis*
NI206-2	*An. punctipennis*	0.67	*An. punctipennis*
NI211-1	*An. punctipennis*	0.67	*An. punctipennis*
NI203-6	*An. punctipennis*	0.57	*An. punctipennis*
NI206-5	*An. punctipennis*	0.57	*An. punctipennis*
NI206-13	*An. punctipennis*	0.50	*An. punctipennis**
NI216-16	*An. punctipennis*	0.50	*An. punctipennis*
NI216-13	*An. punctipennis*	0.44	*An. punctipennis*
NI216-15	*An. punctipennis*	0.44	*An. punctipennis*
NI151-5	*An. punctipennis*	0.44	*An. punctipennis*
NI177-2	*An. punctipennis*	>0.40	*An. punctipennis*
NI177-1	*An. punctipennis*	>0.40	*An. punctipennis*
NI211-4	*An. punctipennis*	>0.40	*An. punctipennis*
ALL LP-2	*An. punctipennis*	0.40	*An. punctipennis*
NI136-1	*An. punctipennis*	0.40	*An. punctipennis*
NI136-4	*An. punctipennis*	0.40	*An. punctipennis*
NI216-7	*An. perplexens*	0.33	*An. punctipennis**

2.3.6. Anopheles quadrimaculatus s.l.

Until recently, *An. quadrimaculatus s.l.* was known as a single species in eastern North America from Texas and Florida to southern Ontario and Quebec (Figure 1.12). *Anopheles quadrimaculatus s.l.* is now recognized as a complex of five sibling species, *An. diluvialus, An. inundatus, An. maverlius, An. smaragdinus,* and *An. quadrimaculatus s.s.* (Reinert *et al.* 1997). However, because examination of the morphological structures used to distinguish the species of this complex required a considerable amount of time, only a subset of all *An. quadrimaculatus s.l.* specimens collected was identified to the species level.

Larvae of *An. quadrimaculatus s.l.* are easily distinguished from those of all other anophelines present in Canada by clypeal setae 2-C, the bases of which are separated from each other by a distance greater than or equal to the diameter of the cuticular pits (also called alveoli) from which they originate in *An. quadrimaculatus s.l.*, and by less than the diameter of one cuticular pit in all other species (Figure 2.26). In addition to setae 2-C, *An. quadrimaculatus s.l.* larvae can be distinguished from those of other anophelines based on abdominal setae 1-II (known as "float hairs"), which are usually partially/fully palmate only in *An. quadrimaculatus s.l.* (*pers. obs.*) (Figure 2.27). Thus, this trait has been incorporated into the character matrix (Table 2.1). The float hairs in *An. quadrimaculatus s.l.* larvae appear to become more palmate as they progress through the fourth instar stage, i.e., they are barely palmate upon molting into the fourth instar and fully palmate before the final molt into the pupal stage.

The adults *An. quadrimaculatus s.l.* have both wings and palpi entirely dark-scaled, wings with aggregations of scales forming a pattern of spots (Figure 2.28a), characteristics shared only with *An. freeborni*, from which it is easily distinguished based on collection location (see above). However, they can also be separated based on the shape of the scales located at the base of the cubital wing vein, which are oval-shaped and rounded apically in *An. quadrimaculatus s.l.* (Figure 2.28b).

Because *An. quadrimaculatus s.l.* is now known as a complex of five sibling species in the USA, distinguishable based on minor morphological difference in the larval and adults stages (Figure 2.10), and the predicted range of sibling species *An.*

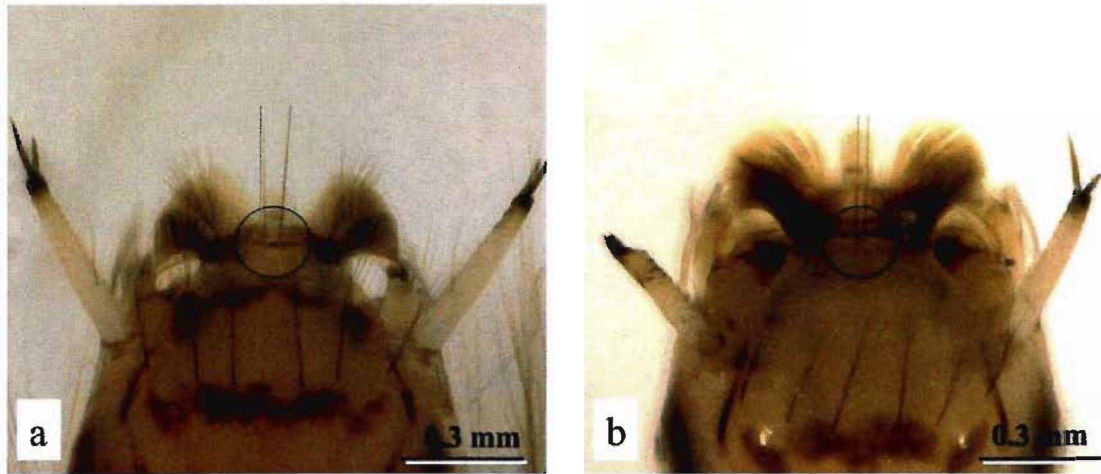

Figure 2.26. Alveoli of setae 2-C in *An. quadrimaculatus s.l.* and *An. punctipennis* (dorsal view). Circles indicate alveoli of setae 2-C, which are separated by a distance greater than the diameter of one alveolus in *An. quadrimaculatus s.l.* larvae, and separated by a distance less than the diameter of one alveolus in the larvae of all other anophelines present in Canada, as in *An. punctipennis* (b).

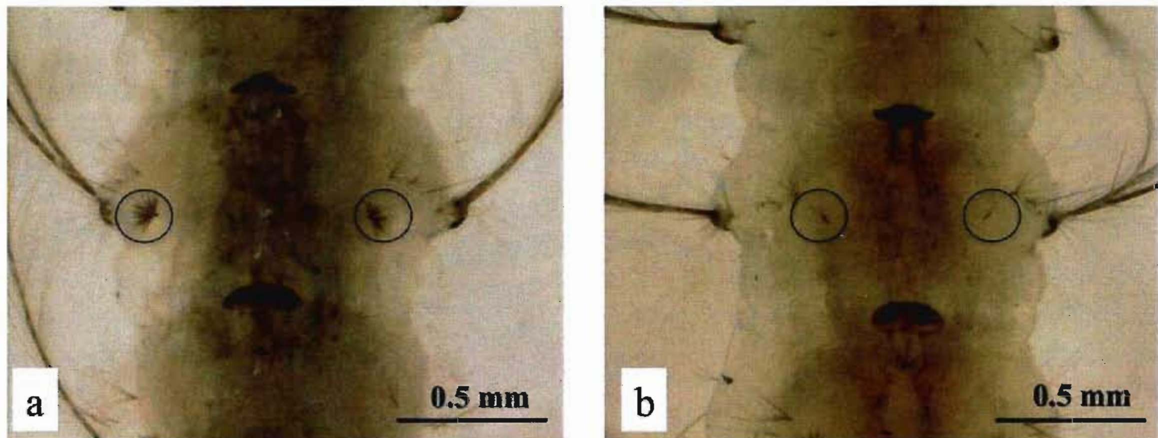

Figure 2.27. Abdominal setae 1-II of *An. quadrimaculatus s.l.* and *An. walkeri* larvae (dorsal view). Larvae of *An. quadrimaculatus s.l.* usually have abdominal setae 1-II partially or fully palmate (a), unlike all other anophelines in Canada, with setae 1-II flattened into leaflets or branched from base, as in *An. walkeri* (b).

smaragdinus approaches southern Ontario, the characters used to identify members of the complex were examined for evidence of cryptic species within this taxon in Canada. A subsample of larval (n=79) and adult female (n=160) *An. quadrimaculatus s.l.* specimens was identified to sibling species level using the key of Darsie and Ward (2005).

Of 79 *An. quadrimaculatus s.l.* larvae, most were identified as *An. quadrimaculatus s.s.*, but a significant proportion were identified as *An. smaragdinus,* some as *An. diluvialus*, and one as *An. maverlius* (Table 2.6). The number of specimens identified as *An. diluvialus* and *An. maverlius* is relatively small, possibly representing abnormalities within *An. quadrimaculatus s.s.* However, the proportion of specimens identified as *An. smaragdinus* was much higher (~37%). Combined with its distribution predicted to approach the Canadian border (Figure 1.16), these data suggested the potential presence of this cryptic species in southern Ontario.

Identification of 160 *An. quadrimaculatus s.l.* adult females to the sibling species level using the key of Darsie and Ward (2005) resulted in a similar pattern to that observed for *An. quadrimaculatus s.l* larvae. Most specimens were identified as *An. quadrimaculatus s.s.* (72.5%), but other members of the sibling species complex were also identified, including *An. smaragdinus* (25.6%), *An. diluvialus* (1.3%), and *An. inundatus* (0.6%) (Table 2.7). The number of specimens identified as *An. diluvialus* and *An. inundatus* was small enough to be considered possible examples of abnormalities within *An. quadrimaculatus s.s.*, but the relatively high number of *An. smaragdinus* specimens, again, suggested the possible occurrence of this member of the *An. quadrimaculatus* sibling species complex in Canada. Therefore, alternative methods of identification, such as egg morphology (see below) or molecular data (see Chapter Three), were also conducted in an attempt to confirm the presence of this species in Canada.

2.3.7. Anopheles walkeri

In the larval stage, *An. walkeri* is easily confused with other anophelines using the key in Wood *et al.* (1979), as the character used to distinguish it from the remaining species usually requires slide mounting of specimens and examination using a compound

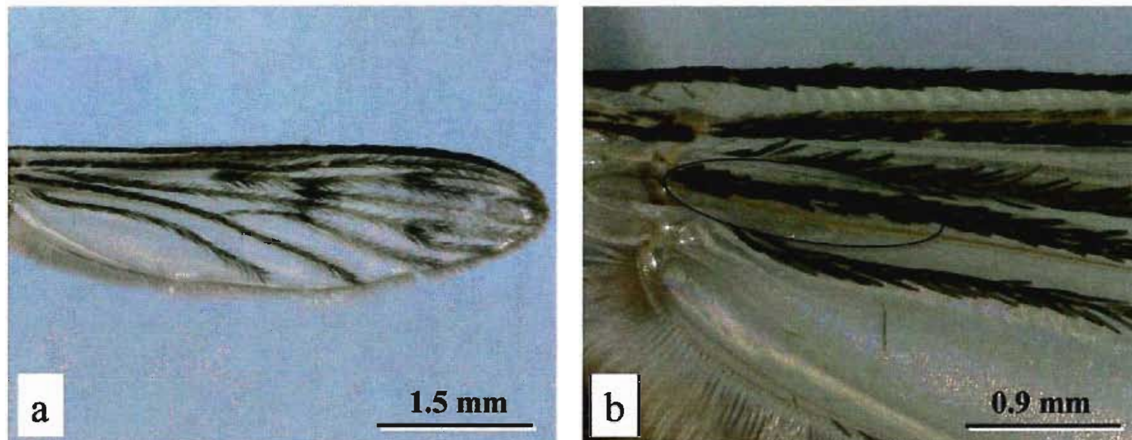

Figure 2.28. Distinguishing characteristics of *An. quadrimaculatus s.l.* adults. Legs, palpi, and wings entirely dark-scaled, wing with a pattern of spot, similar to those of *An. freeborni* except for basal scales of the cubital vein, which are oval-shaped and rounded apically in *An. quadrimaculatus s.l.*, indicated by the circle in (b), and more linear and truncate apically in *An .freeborni* (Figure 2.22b).

Table 2.6. Number and relative proportion of *An. quadrimaculatus s.l.* sibling species (larvae) identified using the key of Darsie and Ward (2005). Specimens collected from southern Ontario, mainly in Niagara, but some from Ottawa and Windsor regions as well.

	Number	% Total
An. quadrimaculatus s.s.	42	53.2
An. smaragdinus	29	36.7
An. diluvialis	7	8.9
An. maverlius	1	1.3
An. inundatus	0	0.0
Total	79	

microscope (i.e., abdominal setae 0-IV and 0-V with multiple branches and apex of clypeal seta 2-C aciculate). In particular, setae 2-C are minutely plumose at the apex in *An. walkeri*, but simple in *An. punctipennis*, which is sometimes visible with a dissecting microscope (Figure 2.23), but not usually. Therefore, it is difficult to distinguish these two species based only on setae 2-C. However, Darsie and Ward (2005) include another character which is useful in separating the larvae of *An. walkeri* from those of all other Canadian anophelines, i.e., seta 1-P is well-developed with 3-5 branches in *An. walkeri*, and weak, single, or branched in outer half in all others (Figure 2.24). Thus, this character has also been included in the *Anopheles* larvae character matrix (Table 2.1).

Adults of *An. walkeri* are easily distinguished from most other anopheline species that occur in Canada by narrow bands of pale scales present at the apices of the second to fifth palpomeres (Figure 2.29). Although the palpi of *An. crucians* also have pale-coloured bands, they also have pale-coloured wing scales (Figure 2.18b), which are entirely dark-scaled in *An. walkeri* (Figure 2.17b). Overall, very few larvae of *An. walkeri* were collected for morphological analysis, and identification of adult males and females did not reveal any differences that might suggest the potential presence of cryptic species within this taxon in Canada.

2.3.8. SEM Egg Data

Egg morphology is often among the first morphological differences to be recognized when comparing specimens of closely related species, and can be used to distinguish suspected cryptic species, *An. perplexens* and *An. smaragdinus*, from isomorphic species known from Canada (*An. punctipennis* and *An. quadrimaculatus s.s.*, respectively). Therefore, eggs were obtained from wild-caught females and examined using scanning electron microscopy. In 2008, a total of 67 blood-fed *Anopheles* females were obtained, and, of those, 39 either died before oviposition or resorbed their eggs. Resorption of eggs can occur in mosquitoes when a suitable oviposition site is not available (Clements 1992). Of the 28 egg batches (or IPBs) obtained, only half (n=14) resulted in SEM images of eggs of sufficient quality for analysis due to initial difficulty in preparing the eggs for SEM analysis.

Table 2.7. Number and relative proportion of *An. quadrimaculatus s.l.* sibling species (adults) identified using the key of Darsie and Ward (2005). Specimens collected from southern Ontario, mainly Niagara, but some from Ottawa and Windsor regions.

	Number ID	% Total
An. quadrimaculatus s.s.	116	72.5
An. smaragdinus	41	25.6
An. diluvialis	2	1.3
An. maverlius	0	0.0
An. inundatus	1	0.6
Total	160	

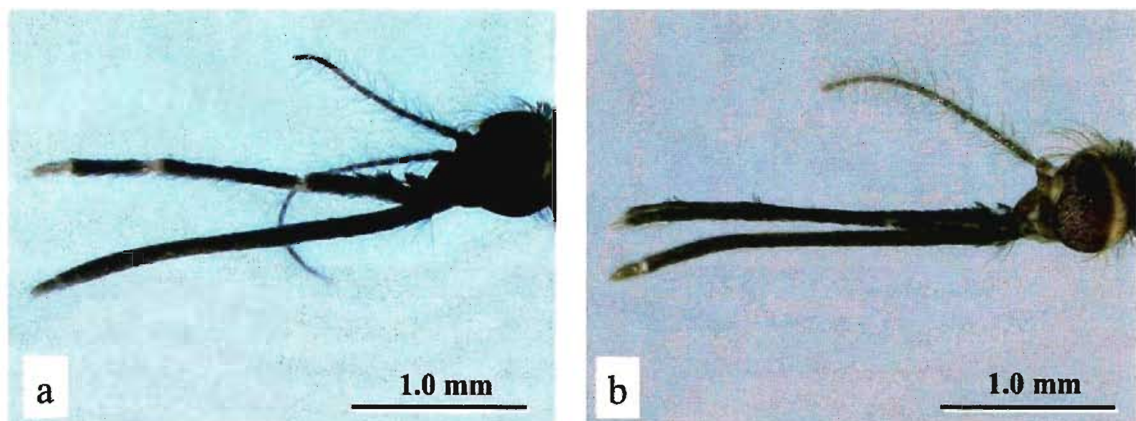

Figure 2.29. Palpi of *An. walkeri* and *An. quadrimaculatus s.l.* (lateral view). Apices of palpomeres 2-5 with pale scales in *An. walkeri* (a), while adults of other anophelines from Canada (except *An. crucians s.l.*) have uniformly dark-scaled palpi, as in *An. quadrimaculatus s.l.* (b).

The chemical method employed for egg preparation resulted in shriveled and brittle eggs that were too damaged to warrant examination with SEM, but eggs not subjected to fixation procedures resulted in much better quality images. While poor in quality, images that allowed species identification of the eggs were obtained for 7 egg batches that had been preserved using the chemical preservation method. However, the images of non-preserved eggs obtained for the other 7 egg batches were of much better quality, easily allowing identification to species, and sibling species in the case of *An. quadrimaculatus s.l.* specimens.

In 2009, fewer blood-fed *Anopheles* females were collected (n=60), but a greater proportion of those (62% in 2009 versus 42% in 2008) successfully oviposited in the lab (n=37). Fresh eggs were used for SEM imaging for 26 IPBs in 2009, all but two of which resulted in good quality images that permitted identification of the eggs to the species level.

Overall, SEM images were obtained from the eggs of 26 females identified morphologically as *An. punctipennis* and one as *An. perplexens* (Table 2.6). Although most *An. punctipennis* females had SCP ratios of 0.5 or more, similar to the results of Fritz *et al.* (1991), 6 (23%) had intermediate SCP ratios, i.e., between 0.33 and 0.5. However, the eggs of all *An. punctipennis* IPBs obtained, as well as those of the female identified morphologically as *An. perplexens* (NI216-7), were identified as *An. punctipennis* (Table 2.6), with wide decks and plastron not visible between the deck and float (Figure 2.30a). There were two cases in which the plastron was slightly visible between the deck and float (Figure 2.30b), but not nearly as much as in *An. perplexens* (Fig. 2.30c), and decks were wider than in *An. perplexens* (Figures 2.30b and 2.30c).

Although eggs from field-collected *An. quadrimaculatus s.l.* females were also to be examined by SEM for evidence of potential cryptic species *An. smaragdinus*, even fewer specimens were obtained. A total of 34 blood-fed *An. quadrimaculatus s.l.* females were collected from NI, BP, and LP, and eggs obtained from 15 of them. Good quality SEM images were obtained for a total of 11 IPBs, and most were identified as *An. quadrimaculatus s.s.* (Table 2.8). However, eggs of four IPBs could not be identified to sibling species level because the diagnostic character (i.e., basal tubercles) was not visible in the SEM images. Eggs were identified as *An. quadrimaculatus s.s.* if the number of

tubercles located at the anterior or posterior end totaled six or more, and as *An. smaragdinus* if five or less (Figure 2.31a). Sometimes eggs attached to SEM stubs on a slight angle, making counting of all the tubercles at either end impossible (Figure 2.31b). Again, an insufficient number of *An. quadrimaculatus s.l.* IPBs was obtained for SEM analysis and, egg morphology could not, therefore, confirm the presence or absence of cryptic species *An. smaragdinus* in Canada.

2.4. Conclusions and Summary

To identify larval and adult *Anopheles* specimens, the identification key for North American mosquito species (Darsie and Ward 2005) was used instead of that for Canadian species (Wood *et al.* 1979), to ensure that species currently known only from the USA, and newly introduced *An. crucians s.l.,* were not overlooked. Based on the morphological characters described in this key, many specimens were identified as *An. perplexens* and *An. smaragdinus*, suggesting their potential presence in Canada.

All *An. perplexens*- and *An. punctipennis*-type specimens (those with pale wing scales that extend to the costa) were identified as either *An. punctipennis* or *An. perplexens* based on subcostal pale spot and preapical dark spot lengths. *An. perplexens*-type females were collected from the three main regions in Ontario, which, interestingly, is where known alvars or alvar-type habitats exist, i.e. NI, OT and NO. *An. perplexens*-type females were not obtained in the other two main collecting regions (WE and BC), despite similar sampling effort and sizes in these locations. The presence of alvars near collecting sites in BC was not determined, and only one is known from the WE area, but is located on Pelee Island, where very limited sampling was conducted only once.

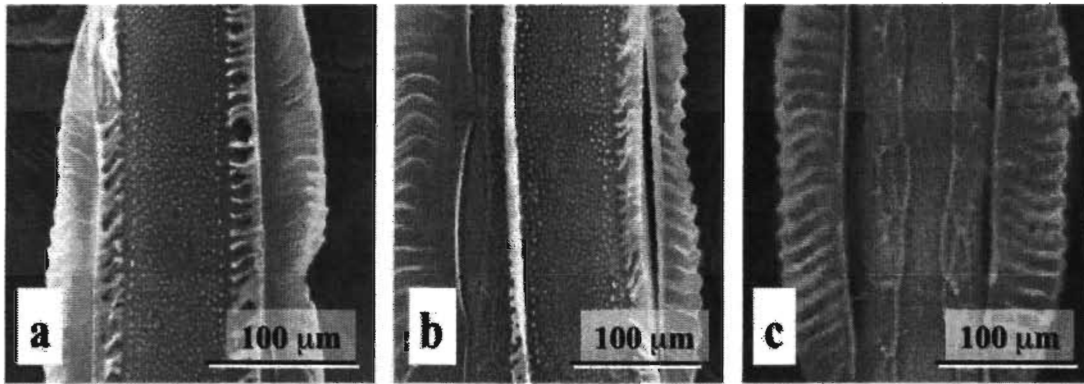

Figure 2.30. Eggs of *An. punctipennis* and *An. perplexens* (ventral view). Plastron cells are not visible between deck and float in eggs of *An. punctipennis* (a), but they are visible between the deck and float in eggs of *An. perplexens* (c, Fig. 2 from Linley and Kaiser 1994). The eggs of one female identified morphologically as *An. perplexens* had some plastron cells visible between the deck and the float (b), but not as much as in *An. perplexens* (c), and deck was wider, as in *An. punctipennis* (a).

Table 2.8. Identifications of *An. quadrimaculatus s.l.* females that produced IPBs, including number of tubercles and egg identifications.

Code	Parental Female	Basal Tubercles	Egg Identification
NI206-11	*An. quadrimaculatus s.s.*	>6	*An. quadrimaculatus s.s.*
NI206-3	*An. quadrimaculatus s.s.*	?	*An. quadrimaculatus s.l.*
NI206-17	*An. quadrimaculatus s.s.*	?	*An. quadrimaculatus s.l.*
NI206-7	*An. quadrimaculatus s.s.*	?	*An. quadrimaculatus s.l.*
NI216-14	*An. quadrimaculatus s.s.*	>6	*An. quadrimaculatus s.s.*
NI203-2	*An. quadrimaculatus s.s.*	>6	*An. quadrimaculatus s.s.*
NI206-1	*An. quadrimaculatus s.s.*	>6	*An. quadrimaculatus s.s.*
NI159-1	*An. quadrimaculatus s.l.*	?	*An. quadrimaculatus s.l.*
NI158-3	*An. quadrimaculatus s.l.*	>6	*An. quadrimaculatus s.s.*
NI158-2	*An. quadrimaculatus s.l.*	>6	*An. quadrimaculatus s.s.*
NI172-1	*An. quadrimaculatus s.l.*	>6	*An. quadrimaculatus s.s.*

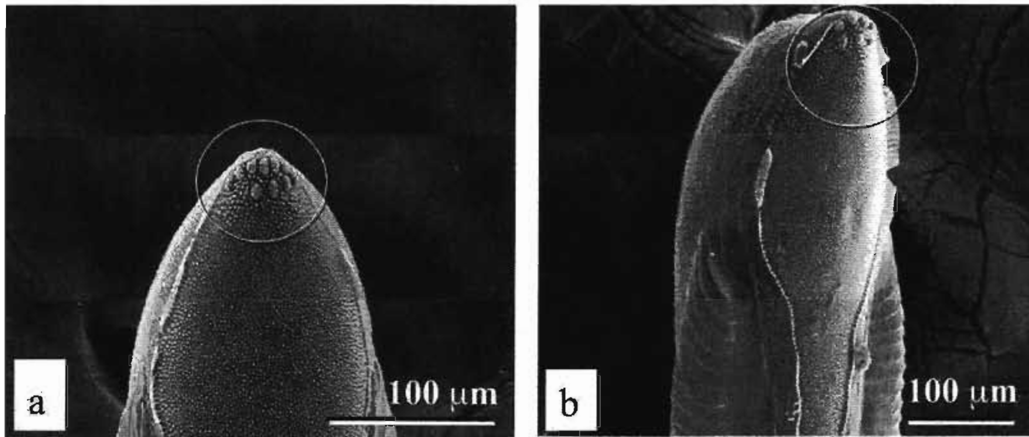

Figure 2.31. Eggs of *An. quadrimaculatus s.s.* (ventral view). *An. quadrimaculatus s.s.* and *An. smaragdinus* can be distinguished based on the number of posterior basal tubercles, which total six or more in *An. quadrimaculatus s.s.* (a) and five or less in *An. smaragdinus*. However, species identification of *An. quadrimaculatus s.l.* eggs was difficult when placed on SEM stubs at an angle (b), which prevented the counting of all tubercles.

With respect to *An. quadrimaculatus s.l.*, and the potential presence of *An. smaragdinus*, specimens collected from southern Ontario were identified to the species level. Among both larvae and adults, few specimens were identified as the more southerly members of the complex (i.e. *An. diluvialus*, *An. inundatus*, and *An. maverlius*), but a significant proportion was identified as *An. smaragdinus*, the sibling species whose range most closely approaches southern Ontario.

Since both *An. perplexens* and *An. smaragdinus* can be reliably identified on the basis of egg morphology, SEM analysis of eggs obtained from females collected in southern Ontario was conducted for evidence of these or other cryptic species. Unfortunately, a sufficient sample size of eggs from *An. perplexens*- or *An. smaragdinus*-type females was not obtained, and the presence of these species in Canada based on morphological data could not be confirmed.

In addition to *An. punctipennis* and *An. quadrimaculatus s.l.*, detailed morphological examination for evidence of cryptic species was conducted for *An. earlei* as well. Many *An. earlei* larvae were observed with additional ATPs on abdominal tergites III-VII, the diagnostic character for *An. freeborni* in both available keys (Darsie and Ward 2005; Wood *et al.* 1979). Examination of this trait in *An. earlei* revealed high levels of variation, with approximately equal amounts without spots (0 additional ATPs), fully spotted (8 or more additional ATPs), or an intermediate number (1 to 7 additional ATPs), with no obvious geographic pattern.

In addition to *An. earlei* and *An. freeborni* in Canada, additional ATPs are also present in other Nearctic members of the *An. maculipennis* complex, including *An. occidentalis* and *An. aztecus* (Pratt 1952), as well as the Palearctic type species of the complex, *An. maculipennis* (Linton *et al.* 2003). However, the use of ATPs as a distinguishing character for the identification of *An. freeborni* larvae in both Wood *et al.* (1979) and Darsie and Ward (2005), with no mention of its occurrence in *An. earlei* larvae, may cause confusion for Canadian identifiers, particularly in British Columbia where both species occur.

In conclusion, morphological examination of larval and adult anophelines collected from across Canada revealed high levels of variation in some traits, suggesting the potential presence of cryptic species within some native taxa, and relative morphological uniformity in others. *An. barberi*, *An. freeborni*, and *An. walkeri* are all known from relatively smaller distributions in Canada, and within each species, no obvious morphological variations were observed. This suggests that *An. barberi*, *An. freeborni*, and *An. walkeri* likely represent single species throughout their range in Canada.

However, larvae and adults of *An. earlei*, *An. punctipennis*, and *An. quadrimaculatus s.l.* exhibited high levels of morphological variation, suggesting that cryptic species *An. perplexens*, *An. smaragdinus*, or others might be present in Canada. Variation in the number of additional ATPs among *An. earlei* larvae collected from across Canada was quantified, and those with and without additional ATPs were collected from all main collection regions from where a sufficient number of specimens was collected for analysis. Whether this trait is indicative of a cryptic species within this taxon is unclear, but if so, it is likely due to ecological (e.g., larval habitat) differences as opposed to geographic location.

Morphological variation among larvae and adults of *An. punctipennis* and *An. quadrimaculatus s.l.* suggested the potential presence of cryptic species *An. perplexens* and *An. smaragdinus*, respectively. While larval and adult characters for *An. perplexens* are known to be unreliable in some cases, so too are those described for *An. smaragdinus*, with features used in the larval and adults keys successfully distinguishing from 80-100% of specimens, depending on the character and sibling species in question (Reinert *et al.* 1997). Since egg morphology is a more reliable method of species identification in the case of *An. punctipennis* and *An. perplexens*, and is also useful for members of the *An. quadrimaculatus* sibling species complex, an attempt to examine the eggs of field-collected specimens was made. Unfortunately, a sufficient number of IPBs was not obtained from *An. perplexens*- and *An. smaragdinus*-type females to confirm their presence or absence in Canada. Because cryptic species can often be recognized based on other types of data, molecular (see Chapter Three) and ecological (see Chapter Four) data were also examined for evidence of cryptic anopheline species in Canada.

Chapter 3: Comparison of ribosomal and mitochondrial DNA in *Anopheles* (Diptera: Culicidae) mosquitoes from Canada

3.1. Introduction to molecular systematics

Anopheles mosquitoes are known for both strong morphological similarity among species, and pronounced morphological variation within species (Krzywinski and Besansky 2003). Isomorphic species complexes are common in *Anopheles* and the members of anopheline species complexes often differ in ecological or behavioural characteristics, which affect their ability to transmit disease (Collins and Paskewitz 1996). The discovery that members of cryptic species complexes can differ in their ability to transmit disease has driven development of alternative methods for reliable identification of *Anopheles* species, particularly in regions where malaria is endemic (Krzywinski and Besansky 2003). Polytene chromosome studies have revealed species-specific banding patterns that have been used successfully to elucidate the members of species complexes in many medically important groups of *Anopheles* mosquitoes (e.g., Frizzi 1947; Kreutzer and Kitzmiller 1971a; Coluzzi *et al.* 1977; Green and Baimai 1985; Foley and Bryan 1991; Ramirez and Desson 2000). However, this cytological technique has major limitations: a) it requires considerable expertise; b) not all *Anopheles* species have polytene chromosomes with reliable banding patterns; c) some species have been identified based on mating incompatibility that did not differ in chromosomal banding patterns (Collins and Paskewitz 1996); and d) it is applicable only to certain life stages and sexes (i.e., fourth instar larvae and/or gravid females).

Advances in other types of genetic techniques during the first half of the 20th century led to the development of biochemical-based assays, such as the analysis of species-specific enzymes by gel electrophoresis, which remained widely used in insect systematics until the 1980s (Berlocher 1984). Since then, development of the polymerase chain reaction (PCR) method and automated sequencing to provide genomic DNA sequences for analysis has led to new techniques that can be used: a) to identify species; b) elucidate cryptic members of species complexes; c) examine population structure within species; and d) establish phylogenetic relationships (Caterino *et al.* 2000, Roe and Sperling 2007). Common molecular methods applied to the identification of mosquito

species include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and PCR amplification and sequencing of genomic DNA (reviewed in Munstermann and Conn 1997; Walton *et al.* 1999b).

RAPD uses multiple primers to randomly amplify sections of the genomic DNA (gDNA) followed by gel electrophoresis of the resulting DNA fragments to visualize differences among species, populations, etc. RFLP involves PCR amplification of gDNA followed by digestion with one or more restriction enzymes to cleave it into smaller DNA fragments, which are then run on a gel to detect species- or population-specific patterns. In recent decades, the use of PCR amplification and sequencing of specific DNA sequences for species identification and phylogenetic analysis has become the method of choice for many researchers and molecular markers are commonly used in studies of insect systematics (reviewed in Munstermann and Conn 1997; Walton *et al.* 1999b; Caterino *et al.* 2000; Krzywinski and Besansky 2003).

Molecular methods for species identification are useful because they require a small amount of template DNA, which permits retention of specimens for use in other types of analyses or to be kept as vouchers (Krzywinski and Besansky 2003). Unlike morphological and cytological methods, molecular methods are applicable to all sexes and life stages, which allows for the identification of isomorphic, immature and other difficult specimens. Through comparison of the nucleotide composition of certain genes or DNA sequences, differences among species, populations, and even individuals can be determined. Fairly long stretches of DNA (up to ~1000bp) are now easily sequenced, allowing nucleotide differences to be easily detected manually or using computer software programs, many of which are available online for free (e.g., MEGA, Clustal, BioEdit). While morphological identification is often the fastest and least expensive method of identifying many *Anopheles* species (Krzywinski and Besansky 2003), it requires taxonomic expertise and many species are difficult to identify based on morphology alone. Therefore, the use of molecular markers for identification at and below the species level has increased dramatically in the field of mosquito systematics (reviewed in Munstermann and Conn 1997; Walton *et al.* 1999b; Caterino *et al.* 2000; Krzywinski and Besansky 2003).

The primary molecular markers used to identify species and elucidate cryptic members of species complexes within Anophelinae include the mitochondrial genes (mtDNA) cytochrome *c* oxidase I and II (COI and COII), as well as the nuclear ribosomal DNA (rDNA) internal transcribed spacer sequence 2 (ITS2). These regions are useful because they contain highly variable regions flanked by highly conserved regions for which universal primers have been designed to amplify and sequence the variable, and often species-specific, sequences located between them (Lunt *et al.* 1996; Collins and Paskewitz 1996). The members of many cryptic anopheline species complexes or groups are distinguishable based on one or more of these markers, such as the *Anopheles barbirostris* subgroup in southeast Asia (Paredes-Esquivel *et al.* 2009), the *Anopheles gambiae* species complex in Africa (Paskewitz *et al.* 1993), the *An. maculipennis* group in Europe (Proft *et al.* 1999), and the *Anopheles quadrimaculatus* complex in southeastern North America (Cornel *et al.* 1996), all of which include major malaria vectors in the regions where they occur, except in North America where malaria has been eradicated since the 1950s (Zucker 1996).

The mitochondrial DNA gene COI has been the focus of many investigations involving insect systematics including taxonomic, population and evolutionary studies (Lunt *et al.* 1996). Its potential for use as a species identification tool across a broad range of taxa (including Insecta) has been well established through the Barcoding of Life Project (Hebert *et al.* 2003a, Hebert *et al.* 2003b). In addition, different portions of the ~1500bp COI gene have been used in phylogenetic analyses of various *Anopheles* species (Mitchell *et al.* 2002; Sallum *et al.* 2002; Foley *et al.* 2007; Paredes-Esquivel *et al.* 2009).

However, analyses using rDNA sequences, particularly ITS2, have become the primary molecular markers used for studies involving the taxonomy and phylogenetics of anophelines since Collins and Paskewitz (1996) reviewed its use to differentiate among cryptic *Anopheles* species. Many cryptic members of *Anopheles* species complexes species have since been identified using ITS2, including the *Anopheles dirus* complex in Thailand (Walton *et al.* 1999a), the *Anopheles annularis* group in southern Asia (Walton *et al.* 2007a), the *Anopheles barbirostris* subgroup in southeast Asia (Paredes-Esquivel *et al.* 2009), the *Anopheles maculatus* group in southeast Asia (Walton *et al.* 2007b), and the *Anopheles maculipennis* complex in Europe (Proft *et al.* 1999), to name just a few. In

North America, similar studies using ITS2 have elucidated the members of species complexes such as the *Anopheles crucians* (Wilkerson *et al.* 2004) and *Anopheles quadrimaculatus* (Cornel *et al.* 1996) complexes in the southeastern United States, and sibling species *An. freeborni* and *Anopheles hermsi* in the western US (Porter and Collins 1991). Although dozens of studies have concentrated on the ITS2 sequences of more than 30 anopheline species, very few instances of intraspecific variation have been observed (but see Onyabe and Conn 1999; Wilkerson *et al* 2004; Fairley *et al.* 2005; Li and Wilkerson 2007).

While the use of COI and ITS2 for molecular analyses of *Anopheles* has dominated the literature over the past two decades, studies involving the use of ITS1 are not common. However, ITS1 sequences are known to vary extensively in both sequence and length (LaRue *et al.* 2009) and have been used successfully to identify cryptic species within other groups, including insects. Analysis of ITS1 sequences in the jellyfish *Aurelia aurita* (Cnidaria, Scyphozoa) revealed the presence of cryptic species now recognized as members of a species complex that exhibit reproductive isolation despite a lack of physical barriers to gene flow (Dawson and Jacobs 2001). Examination of ITS1 sequences within species of biting flies (*Culicoides spp.*) revealed species-specific differences, as well as evidence that one population of *Culicoides impunctatus* was genetically more similar to a related species, *Culicoides imicola* (Ritchie *et al.* 2004). A study of the ITS1 rDNA in 15 species from five genera of black flies found high levels of intraspecific variation in this region, indicating its potential for population-level studies of *Simuliium spp.* (LaRue *et al.* 2009).

Although few authors have examined ITS1 sequences in mosquitoes, preliminary results indicate that, unlike the ITS2 region, ITS1 contains considerable intraspecific and/or intraindividual variation (Wesson *et al.* 1992; Paskewitz *et al.* 1993; Fairley *et al.* 2005; Bower *et al.* 2008, Bower *et al.* 2009), making its analysis more complicated. The ITS1 sequences of *Anopheles* species whose ranges extend into Canada, or related Palearctic members of the *An. maculipennis* complex, have not yet been published.

For molecular data to be useful in taxonomic and phylogenetic comparisons across a wider range of taxa, and to be able to make sense of the vast amounts of molecular data being generated by research groups around the world, the need for

consistency in the type of sequence data obtained through molecular investigations is becoming increasingly apparent (Caterino *et al.* 2000).

Therefore, for this thesis I chose COI and ITS2 (commonly used in investigations of anopheline systematics), as well as ITS1 (potentially informative beyond the species level), as the molecular markers to examine Canadian anophelines. COI, ITS2, and ITS1 sequences from *An. barberi, An. earlei, An. freeborni, An. punctipennis, An. quadrimaculatus s.l.,* and *An. walkeri* specimens collected from various geographic regions throughout their range in Canada were examined for evidence of cryptic species near the northern limits of these species' ranges.

3.1.1. Mitochondrial COI

Mitochondrial DNA are small, circular, extrachromosomal DNA molecules that are present in hundreds of copies inside mitochondria and, thus, are inherited from the female parent only. The mitochondrial genes COI and COII are among the most widely used molecular markers for studies of insect taxonomy and phylogenetics, particularly COI (Lunt *et al.* 1996; Hebert *et al.* 2003a; Roe and Sperling 2007). The degree of sequence variation within these genes has proven useful for both genus- and species-level investigations (Simon *et al.* 1994). Features of COI that make it particularly suitable for such studies include: a) ease of isolation and high copy number, b) the close association of highly conserved and variable regions within the gene which allows universal primers to amplify and sequence the more variable parts of the COI gene across a wide range of insect taxa, and c) its large size which allows the sequencing of hundreds of nucleotides for comparison (Lunt *et al.* 1996). In 2003, Hebert and colleagues proposed a method for species identification that involved the amplification and sequencing of a standardized 650bp portion of the COI, located at the 5′end of the gene (Figure 3.1), which they called the barcode region (Hebert *et al.* 2003a, Hebert *et al.* 2003b). This led to the development of the International Barcoding of Life Project (http://www.ibolproject.org), a global effort whose aim is to establish DNA barcodes for all species of life on earth and ultimately allow even non-taxonomic experts to identify species rapidly and accurately. The barcoding region of COI has since been used successfully to identify species in many

arthropod groups such as mayflies (Ball *et al.* 2005), spiders (Barret and Hebert 2005), and bees (Sheffield *et al.* 2009), as well as biting flies such as deer and horse flies (Cywinska *et al.* 2010), black flies (Ilmonen *et al.* 2009; Rivera and Currie 2009), and mosquitoes (Cywinska *et al.* 2006).

In addition to species identification, COI barcoding has also been used to elucidate cryptic members in some species complexes, including the butterfly *Astraptes fulgerator* (Hebert *et al.* 2004) and multiple tachinid fly species (Smith *et al.* 2007). While the barcoding region of COI has been used successfully to identify species and members of cryptic species complexes, others caution that there is no single diagnostically informative region within COI, and that other locations downstream from the barcoding region may even be better, depending on the particular group of insects being examined (Roe and Sperling 2007).

In fact, recent studies have shown that other portions of COI located in the 3′ end of the gene may offer more phylogenetically informative characters than the barcoding region located at the 5′ end of the gene (Figure 3.1), such as the cryptic mayfly *Baetis rhodani* species complex (Ephemeroptera, Baetidae) (Williams *et al.* 2006) and the black fly group *Simulium vernum* (Ilmonen *et al.* 2009). A recent study of *An. punctipennis* specimens collected from various geographical locations throughout Vermont showed that the last 450bp at the 3′ end of the COI gene was informative at the population level (Fairley *et al.* 2000).

Cywinska *et al.* (2006) examined the COI barcodes from 37 species of mosquitoes in Canada and established their ability to distinguish species identified based on previous morphological work, including five of the six *Anopheles* species known to occur in Canada based on morphological characters at that time. This study included specimens collected mainly from Ontario, but many species have much broader distribution ranges, including two species whose ranges extend from coast to coast, *An. earlei* and *An. punctipennis.*

Therefore, although some studies using COI have included *Anopheles* species whose ranges extend into Canada, the data are limited primarily to the barcoding region of COI and to specimens collected from Ontario. In their examination of the full COI and COII sequences from a wide variety of insect species (including two *Anopheles* species),

Roe and Sperling (2007) recommend maximizing sequence length to improve the probability of including an informative region within the chosen sequence. Therefore, in the current study, the larger ~800bp fragment located at the 5′ end of the gene was selected as one of three DNA sequences to be examined for evidence of cryptic species within anophelines collected from across Canada.

3.1.2. Ribosomal ITS2 and ITS1

The chromosomal complement of *Anopheles* mosquitoes consists of 3 pairs of chromosomes (2n=6): the X/Y (sex differentiating) chromosomes, and two (autosomal) chromosomes, termed chromosomes 2 and 3 (Beckingham 1982). The highly conserved ribosomal genes, responsible for the formation of ribosomes, are located in heterochromatic regions of the X and Y chromosomes called the nucleolar organizers (NORs) (Marchi and Pili 1994). These rDNA genes are organized into hundreds of repeating units, each consisting of an external transcribed spacer (ETS) and three transcribed rDNA subunits (18S, 5.8S, and 28S) separated by two transcribed spacer sequences (ITS1 and ITS2), with each repeating unit separated by a highly variable non-transcribed spacer sequence called the intergenic spacer (IGS) (Beach *et al.* 1989) (Figure 3.2).

The rDNA genes produce rRNA molecules that are involved in the formation of ribosomes and, due to functional constraints, are highly conserved even among distantly related organisms (Hillis and Dixon 1991). Apart from some short homologous domains involved in rRNA processing (Hillis and Dixon 1991; Wesson *et al.* 1992), the internal transcribed spacers are not functionally constrained and, thus, evolve more rapidly, and can be used to distinguish among even closely related species (Paskewitz and Collins 1996). The highly conserved 18S, 5.8S and 28S genes sequences have allowed the design of universal primers to amplify the highly variable ITS1 and ITS2 sequences between them (Porter and Collin 1991).

ITS2 has become the most widely used molecular marker in taxonomic and phylogenetic studies of *Anopheles* mosquitoes since Porter and Collins (1991) published

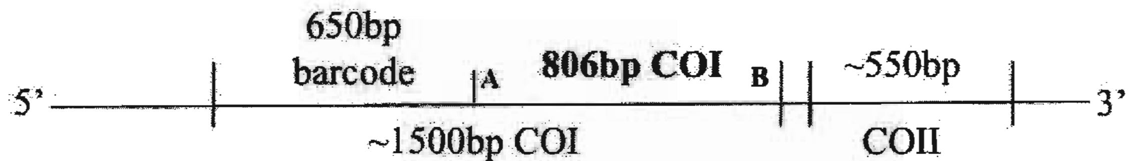

Figure 3.1. Schematic diagram of mtDNA genes COI and COII. The ~650bp barcode region is located at the 5′ end of the COI gene, and the 806bp region used in the current study at the 3′ end. Locations of universal primers binding sites ([A] -forward primer; [B] –reverse primer) are shown (adapted from Roe and Sperling 2007).

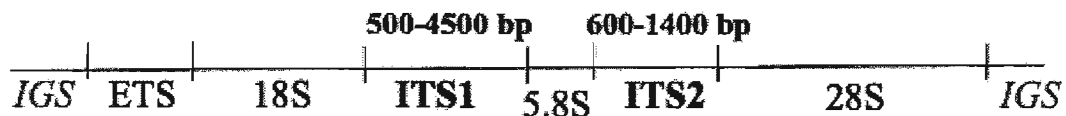

Figure 3.2. Schematic diagram of rDNA subunits and spacer sequences. Highly variable ITS1 and ITS2 regions are flanked by highly conserved 18S, 5.8S, and 28S rDNA subunits (adapted from Hillis and Dixon 1991).

the primers that amplified species-specific ITS2 sequences of two isomorphic anopheline species (for examples see Paskewitz *et al.* 1993; Cornel *et al.* 1996; Marinucci *et al.* 1999; Hackett *et al.* 2000; Walton *et al.* 2007b). In 1996, Collins and Paskewitz reviewed the use of ITS2 to differentiate cryptic *Anopheles* species and outlined several factors that make ITS2 a useful marker for such studies, such as the short length (<1000bp) of most sequences (which makes amplification and sequencing straightforward), and lower level of ITS2 sequence variation within species than between them. In the past two decades, many studies of *Anopheles* mosquitoes have used ITS2 to describe cryptic species, propose phylogenies, and develop PCR assays for molecular identification of isomorphic species (for examples see Fritz *et al.* 1994, Hackett *et al* 2000, Kampen 2005, Linton *et al.* 2003, Malafronte *et al.* 2007, Marinucci *et al.* 1999, Proft *et al.* 1999, Walton *et al.* 1999, Walton *et al.* 2007a, Walton *et al.* 2007b).

Studies of North American anophelines have used ITS2 data to elucidate cryptic members of species complexes, but they used specimens collected only from more southern locations within the USA (Porter and Collins 1991; Cornel *et al.* 1996; Wilkerson *et al.* 2004). Porter and Collins (1991) described species-diagnostic differences in the ITS2 sequences of two morphologically indistinguishable species, *An. freeborni* and *An. hermsi*, from California. These same primers were later used to elucidate the cryptic members of two species complexes in the southeastern United States, the *An. quadrimaculatus* complex (Cornel *et al.* 1996), and the *An. crucians* complex (Wilkerson *et al.* 2004). Therefore, ITS2 was selected as the second of three molecular markers to be examined in the current study for evidence of cryptic species within anophelines collected from across Canada.

The other internal rDNA transcribed spacer sequence, ITS1, has not been as well examined, especially within *Anopheles*. In their analysis of ITS1 sequences in the mosquito *Aedes aegypti*, Wesson *et al.* (1992) showed that both intraspecific and intragenomic variation were present in this species, indicating that ITS1 varies not only among individuals but within them as well. Similar studies have revealed intragenomic variation in ITS1 sequences within some members of the African *An. gambiae* complex (Paskewitz *et al.* 1993), as well as in Australian species *An. farauti* (Bower *et al.* 2008) and the *An. punctulatus* group (Bower *et al.* 2009). Within the *An. gambiae* complex,

multiple ITS1 spacer lengths were prevalent in *An. merus* and *An. melas* (up to 14 variants each), due to varying numbers in a 250bp internal repeat, which results in ITS1 lengths ranging from 1.8 to 5.5kb and 1.25 to 4.5kb, respectively (Paskewitz *et al.* 1993). Australian species *An. farauti* and members of the *An. punctulatus* group revealed similar patterns of ITS1 intraspecific and intragenomic variability due to complex patterns of repeating subunits, from small 21bp repeats to large ~360bp repeats, which result in multiple spacer lengths (Bower *et al.* 2008, Bower *et al.* 2009). Therefore, although ITS1 sequences appear to be longer and more complex than those of ITS2 within *Anopheles*, variation in ITS1 sequences may provide cryptic species-level information not possible using COI or ITS2. Therefore, ITS1 was chosen as the third molecular marker to be examined for evidence of cryptic species within the *Anopheles* species whose ranges include Canada.

3.1.3. Canadian anophelines

Detailed descriptions of the seven species previously known from Canada based on morphological data (Wood *et al.* 1979; Thielman and Hunter 2007) (i.e., *An. barberi, An. crucians, An. earlei, An. freeborni, An. punctipennis, An. quadrimaculatus,* and *An. walkeri*) were provided in Chapter One, as well as other species whose ranges could potentially extend into Canada (i.e., *An. occidentalis, An. perplexens,* and *An. smaragdinus*). Since *Anopheles* mosquitoes are potential vectors of pathogens of medical and veterinary importance, and represent a risk to both human and animal health, accurate identification at and below species level is essential for mosquito surveillance and control programs to be successful. *Anopheles* specimens from Canada have not yet been included in any systematics or phylogenetic analyses using molecular data, except for COI barcoding of specimens from Ontario (Cywinska *et al.* 2006).

Therefore, the objective of this study was to characterize the ITS2, ITS1, and 3′ 800bp COI regions of anopheline specimens collected from locations across Canada and to examine the resulting sequences for molecular evidence of cryptic species here at the northern limits of their ranges.

3.2. Materials and Methods

3.2.1. Mosquito collections and identification

Anopheline larvae and adults were collected from May until October during the 2005-2009 field seasons from a variety of locations throughout Canada (Figures 2.11, 2.12, and 2.13). Methods for the collection, rearing, and preservation of larval and adult specimens were described in Chapter Two. Larvae and adults were identified morphologically to species using the keys of Wood *et al.* (1979) and Darsie and Ward (2005). Specimens used in molecular analyses, and their associated collection data (e.g., species identification, date of collection, and collection location) are listed in Appendix III.

3.2.2. Specimen preparation and DNA extractions

DNA was extracted from single mosquitoes. To prepare larval mosquitoes, the head and abdomen of each larva were separated from the thorax using a clean, sterile scalpel blade and placed in a 2mL cryogenic vial filled with 95% ethanol to retain for morphological verification of molecular species identification. The thorax was either placed in a separate vial of 95% ethanol for use in future molecular studies or placed directly into the lysis buffer solution (Lysis T) of the GeneElute™ Mammalian Genomic DNA Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) and used immediately in the DNA extraction procedure. This DNA extraction kit was known to work well for mosquitoes (A. Cywinska, *pers. comm.*). This method of specimen preparation worked well for *Anopheles* larvae as most diagnostic characters are located on the head and abdomen, and a large amount of genomic (gDNA) for use in molecular analyses is extracted from the thorax.

To prepare adult specimens for molecular analyses, mosquitoes were removed from freezer and three legs (including as much of the coxae as possible) were removed from the rest of the mosquito using clean, sterile forceps and either placed into a cryogenic vial with 95% ethanol for future use or placed directly into the tissue lysis

solution of the GeneElute™ DNA extraction kit and used immediately in the DNA extraction procedure.

The gDNA from adult legs or larval thoraces was eluted into 30μL-40μL of the Sigma GeneElute™ Elution Solution (10mM Tris-HCl. 0.5mM EDTA, pH 9.0) and stored at 4°C for use in PCR reactions within one week, or frozen at -20°C for long term storage and future use.

3.2.3. Primers and PCR Conditions

PCR reactions were prepared the same for all DNA sequences to be amplified (i.e., COI, ITS2, and ITS1) (Table 3.1). The same volumes of reagents were used for all PCR reactions, but the volumes of gDNA and molecular grade water varied depending on the source and age of the gDNA. Extractions from larval thoraces resulted in greater amounts of gDNA compared to those from three adult legs, so less gDNA was required in PCR reactions when from larvae (4.0μL) than from adults (5.0μL). Also, gDNA samples stored for long periods (one year or more) tended to decrease in quality over time, so more gDNA was required for PCR reactions involving older samples (5.0-6.0μL). As all PCR reactions were carried out in a total volume of 25μL, the volume of water used in each reaction was adjusted accordingly. To amplify ~800bp partial COI sequences, the primers C1-J-2195 (Simon *et al.* 1994) and UEA10 (Lunt *et al.* 1996) were used (Table 3.2). PCR conditions for COI reactions were as follows: 95°C for 2min (initial denaturation step), followed by 35 cycles of: 95°C for 30s, 51°C for 45s, 72°C for 1min, then a final extension step at 72°C for 10min. These primers successfully amplified the ~800bp COI region successfully in all specimens examined.

To amplify the ITS2 region, the primers ITS2F and ITS2R (Wilkerson *et al.* 2004) were used (sequences listed in Table 3.2). PCR conditions were as follows: 95°C for 2min (initial denaturation step), followed by 40 cycles of: 95°C for 30s, 61°C for 30s, 72°C for 1min, then a final extension step at 72°C for 10min. The ITS2F and ITS2R primers resulted in single bands from ~300-400bp for all species examined, except *An.*

earlei. Anopheles earlei produced single but much larger (~800bp) ITS2 PCR products than all other species examined in this study.

Table 3.1. Volumes and concentrations of solutions used in preparation of PCR reactions for amplification of COI, ITS2 and ITS1 sequences.

a) PCR Master Mix

Component	Volume
10X Thermopol II Buffer[1]	= 2.30µl
50mM $MgSO_4$	= 1.30µl
10mM Forward Primer	= 0.25µl
10mM Reverse Primer	= 0.25µl
10mM dNTPs[2]	= 0.30µl
Taq polymerase[3]	= 0.30µl
Master Mix	**= 4.70µl**

b) PCR reactions

Component	Volume
Master Mix	= 4.7µl
Sample gDNA[4]	= 4.0-6.0µl
Sterile H_20[5]	= 14.3-16.3µl
Total volume	**= 25.0µl**

[1] 10X Thermopol II (Mg-free) Buffer: 10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl, 0.1% Triton X-100, pH 8.8 at 25°C (New England BioLabs)
[2] dNTPs: 100mM dATP, dCTP, dGTP, and dTTP (GE Healthcare Canada)
[3] Taq polymerase: 5000U/ml (New England BioLabs)
[4] volume of DNA varied depending on source (larva/adult) and age of the gDNA
[5] volume of H_20 adjusted for total volume of 25.0µl

The same primers used to amplify ITS2 fragments were used successfully for sequencing of the resulting fragments in all species examined, again except *An. earlei.* The ITS2F primer successfully sequenced the first ~350bp of the ITS2 sequence until the appearance of a GA dinucleotide repeat, and then the sequence quality becomes too poor to generate a reliable sequence. The ITS2R primer successfully amplified the ITS2 fragment of *An. earlei,* but could not generate a reliable reverse sequence to include in analysis. Therefore, two new primers were designed based on *An. freeborni* ITS2 sequences (a close relative of *An. earlei*; both species belong to the Maculipennis Group), slightly internal to the binding location of ITS2R (i.e., ITS2earlR2a and ITS2earlR2b, Table 3.2).

Both ITS2earlR2a and ITS2earlR2b primers sequenced the first ~200bp in the reverse direction better than the ITS2R, and the GA repeat that interferes with the forward sequencing reaction was visible in the reverse reaction, which allowed the first ~318bp of the forward sequence to be combined with the last ~450bp for a total sequence length of about~780bp. However, the sequence quality of the last ~450bp was often still very poor, due to a series of runs and dinucleotide repeats in this region. Therefore, the 318bp portion located near the 5′ end of the 800bp sequence (the only fully reliable section) was used in analyses of *An. earlei* ITS2 sequences.

To amplify the ITS1 region, two sets of primers were designed based on a combination of sequences obtained both from this study and from Genbank. The first forward ITS1 primer (ITS1F) was based on the 18S sequence of *An. quadrimaculatus* from Genbank (accession number AY988423) and the reverse, complementary sequence for the forward ITS2 primer (ITS2F, located within the 5.8S subunit) was used for the ITS1 reverse primer (ITS1R). These primers successfully amplified single bands from *An. quadrimaculatus* and *An. walkeri* specimens, but no bands were produced for all other species tested (*An. barberi, An. earlei* and *An. punctipennis*). Therefore, the *An. quadrimaculatus* and *An. walkeri* ITS1 sequences obtained with the first set of ITS1 primers were aligned using ClustalW2 sequence alignment software (http://www.ebi.ac.uk/Tools/clustalw2) and new primers were selected where both sequences matched, slightly internal to the first pair (ITS1ver2F and ITS1ver2R). These primers successfully amplified the ITS1 regions in all species examined, except *An.*

Table 3.2. List of primers used to amplify and sequence COI, ITS2, and ITS1 sequences. (* length and ITS1 fragments vary depending on species).

Locus (Genome)	Primers	Sequence	Reference	Seque Leng
COI	C1-J-2195	5′ TTGATTTTTTGGTCATCCAGAAGT 3′	Simon *et al.* 1994	806
(mtDNA)	UEA10	5′ TCCAATGCACTAATCTGCCATATTA 3′	Lunt *et al.* 1996	
ITS2	ITS2F	5′ TGTGAACTGCAGGACACATGAA 3′	Wilkerson *et al.* 2004	300-80
(rDNA)	ITS2R	5′ ATGCTTAAATTTAGGGGGTAGTC 3′	Wilkerson *et al.* 2004	
	ITS2earlR2a	5′ CATGTACTCCCGCAGCTAGG 3′	this study	
	ITS2earlR2b	5′ ATTTGAGGCCCATGTACTCCCG 3′	this study	
ITS1	ITS1(18S)Fd	5′ ATGTGGGTATCAGCGTGTCTCC 3′	this study	500-4500b
(rDNA)	ITS1(5.8S)Rd	5′ ATCGGTGTTCTTCATGTGTCCTC 3′	this study	
	ITS1ver2F	5′ GCAATGGTCCATACGAACTC 3′	this study	
	ITS1ver2R	5′ TTGTGACGCGCATTTAGCT 3′	this study	

barberi, the only species that belongs to the Plumbeus Group instead the Maculipennis Group (Figure 1.6), and thus may contain sufficient nucleotide differences in the 18S and/or 5.8S primer binding sites to go unrecognized by ITS1ver2F, ITS2ver2R, or both.

3.2.4. PCR products and Sequencing

PCR products were stored at 4°C while subsamples of each PCR reaction were separated using gel electrophoresis to ensure successful amplification of the desired sequence. A 4μL aliquot from each 25μL PCR reaction was combined with 4μL of 3X or 6X Loading Dye (Fermentas Canada, Inc) and run on a 1-2% agarose gel containing 0.5-1.0μL ethidium bromide at ~105 volts for about 30min (COI) or 1.5hrs (ITS2 and ITS1). For each row of samples on the gel, 1-3 lanes of appropriate DNA ladder were included to determine the size of PCR fragments: COI – PCR Sizer (100-1,000bp); ITS2 and ITS1– High Ranger (300-10,000bp) or Full Ranger (100-5,000bp) (all from Norgen Biotek Corporation, Inc., St. Catharines, Ontario, Canada). Bands were visualized using UV light and images of the gels were obtained to determine the size of PCR products based on the bands of known size in lanes containing DNA ladders.

For PCR reactions that produced single bands of expected sizes for the molecular marker used, the remaining ~20μL of unpurified PCR products were sent for purification and direct sequencing to Genome Quebec at the McGill University Innovation Centre in Montreal, Quebec.

3.2.5. Consensus sequence determination

All PCR products were sequenced in both directions using the same forward and reverse primers for sequencing that were used to amplify the fragment, except for the ITS2 sequences of *An. earlei,* for which species-specific primers for sequencing were designed (see above). Electropherograms were downloaded from the Genome Quebec website and examined using the DNA sequence editing program Four Peaks version 1.7.2 (A. Griekspoor and Tom Groothuis, mekentosj.com). Those with strong, clear signals and few to no questionable base calls were exported as text files and copied into a Word

document. Forward and reverse sequences for each specimen were aligned using the sequence alignment program, ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html), and any differences between the forward and reverse sequences were analyzed and edited only if a reliable nucleotide could be determined for that location using at least one of the original electropherograms.

In general, most consensus sequences (final sequence after forward and reverse sequences were aligned and the ends had been shortened to the nearest reliable nucleotide) were established using the first half of the reverse sequence and the second half of the forward sequence. Some sequences were poorer in quality and required careful editing throughout the entire length of sequence using the Clustal alignment and original electropherograms. Once all consensus sequences for a given molecular marker were determined, they were aligned using ClustalW2 to determine common start and stop points for each sequence (to allow comparisons both within and between species). The start and stop points selected were the first and last nucleotides in common among all species for a particular marker (to maximize the number of nucleotides available for comparison).

3.2.6. Data Analyses

COI sequences were aligned and analyzed using the molecular evolutionary genetic analysis software program MEGA version 4.0 (Tamura *et al.* 2007). Nucleotide compositions of each sequence were generated and the GC content calculated. Sequence divergences were determined using the Kimura two-parameter (K2P) distance model (Kimura 1980). Neighbour-joining (NJ) trees were built with the K2P model, with pairwise deletion of missing data and inclusion of all codon positions and substitution type, and with branch support assessed by bootstrapping with 500 replicates. A *Bironella gracilis* COI sequence, obtained from Genbank (Accession number AF417725.1), and a *Bironella hollandi* ITS2 sequence (EF619445.1) were used as outgroup species. ITS2 and ITS1 sequences were also analysed manually, using ClustalW2, and MEGA 4.0.

3.3. Results

3.3.1. COI

A total of 224 COI sequences were obtained for analysis, each 806bp in length (specimens summarized by species and collection location in Table 3.3). COI sequences had a strong A+T bias in all species examined (average ~70% for all codons and species), with the average GC content ranging from 29.1% in *An. earlei* and *An. punctipennis* to 30.8% in *An. walkeri* (Table 3.4).

Nucleotide differences among samples identified as *An. punctipennis* and *An. perplexens* were present, but they did not correspond to morphological identifications of specimens. The same was true for specimens identified as either *An. quadrimaculatus s.s.* *An. quadrimaculatus s.l.*, or *An. smaragdinus*. However, original species names (based on morphology) were kept the same to reflect the initial morphological identification of specimens, and to facilitate comparison of sequences from each morphological type.

Levels of intraspecific variation were very low in all species examined, except *An. punctipennis* and *An. walkeri.* Average pairwise sequence divergences ranged from 0.0% (*An. freeborni*) to 0.9% (*An. punctipennis* including those identified as *An. perplexens*), but were much higher (4.6%) in *An. walkeri* (Table 3.5). Since morphological identifications of suspected cryptic species did not correspond to distinct clusters based on molecular results, they were grouped together with their respective isomorphic native counterparts (i.e., *An. punctipennis* and *An. quadrimaculatus s.s.*) for all remaining analyses.

***Anopheles barberi* COI**

All 16 *An. barberi* specimens were collected from the same woodlot in Niagara Falls, Ontario, despite effort to collect this species from other locations within their known range, including Point Pelee National Park in Leamington, ON (Smith and Trimble 1973) and Perth, ON (Wood *et al.* 1979). Intraspecific sequence divergence

Table 3.3. Number of specimens for each species from each collection region for which COI sequences were obtained. Region codes listed in Appendix I.

	BC	NI	WE	NO	OT	LP	NF	RQ	AL	HL	BP	Total
An. barberi		16										16
An. earlei	4			14	8		1	5	8			40
An. freeborni	30											30
An. perplexens		23		5					2	2		32
An. punctipennis	14	23	7								3	47
An. quadrimaculatus s.l.		37	13		2							52
An. smaragdinus		8										8
An. walkeri				6		6						12
Total	48	107	20	25	10	6	1	5	10	2	3	224

Table 3.4. Average base composition (over all codons) of COI for each species examined, including combined GC content.

	T	C	A	G	GC%
An. barberi	39.5	15.5	31.2	13.9	29.4
An. earlei	38.4	14.9	32.4	14.2	29.1
An. freeborni	38.3	15.2	32.3	14.1	29.3
An. perplexens	38.1	15.2	32.8	14	29.2
An. punctipennis	38.1	15.1	32.8	14	29.1
An. quadrimaculatus s.l.	37.8	15.9	31.7	14.6	30.5
An. smaragdinus	37.8	15.9	31.8	14.5	30.4
An. walkeri	38.6	15.9	30.6	14.9	30.8
Average	38.3	15.5	32	14.3	29.7

among the 16 specimens was very low, ranging from 0.0-0.4% with a mean of 0.1%, and with seven haplotypes and 7/806 variable sites (Table 3.5). Neighbour-joining (NJ) analysis did not reveal any significant clusters (Figure 3.3).

Anopheles earlei COI

A total of 40 *An. earlei* sequences were obtained from specimens collected from a wide variety of locations across Canada, including British Columbia, Manitoulin Island (ON), Radisson (QC), and Newfoundland (NF), among others (Table 3.3). K2P sequence divergence ranged from 0-0.6% with an average of 0.2%, with 21 haplotypes and a total of 21/806 variable sites identified among the 40 sequences (Table 3.5). NJ analysis of K2P distances of *An. earlei* COI sequences did not reveal strong support for distinct clusters of specimens based on collection region (Figure 3.4). However, there was weak support for one cluster of specimens (bootstrap value 67%), which included all four specimens from BC plus three from AL, but there were five more specimens from AL that were not included in this cluster (Figure 3.4).

Anopheles freeborni COI

COI sequences were obtained from 30 *An. freeborni* specimens, all collected from British Columbia, the only region in Canada where they occur (Table 3.3). Although sampling was conducted from a wide variety of locations throughout the province, *An. freeborni* is present mainly in the Kootenay region, Oliver, Vernon, and in Kamloops, and most specimens examined were from a pond on a farm in Kamloops in which all three anopheline species known from BC co-occur. Intraspecific variation was lowest in this species, with K2P sequence divergence levels ranging from 0-0.2%, with a mean of 0% (Table 3.5). Only four haplotypes were present among the 30 specimens with even fewer variable sites (3/806). All samples formed a single cluster according to NJ analysis of K2P distances (Figure 3.5).

Table 3.5. Kimura two-parameter (K2P) sequence divergence levels in COI sequences for each species examined. Includes the number of specimens sequenced, the minimum, maximum, and average pairwise sequence divergences, and the number of haplotypes and variable sites found within the COI sequences for each species. Note: suspected cryptic species *An. perplexens* and *An. smaragdinus* were grouped with their isomorphic native counterparts (*An. punctipennis* and *An. quadrimaculatus s.s.*, respectively).

	n	Min	Max	Ave	Haplotypes	Variable Sites
An. barberi	16	0.000	0.004	0.001	7	7
An. earlei	40	0.000	0.006	0.002	21	21
An. freeborni	30	0.000	0.002	0.000	4	3
An. punctipennis/perplexens	79	0.000	0.027	0.009	56	70
An. quadrimaculatus/smaragdinus	60	0.000	0.012	0.006	46	52
An. walkeri	12	0.000	0.082	0.046	10	87

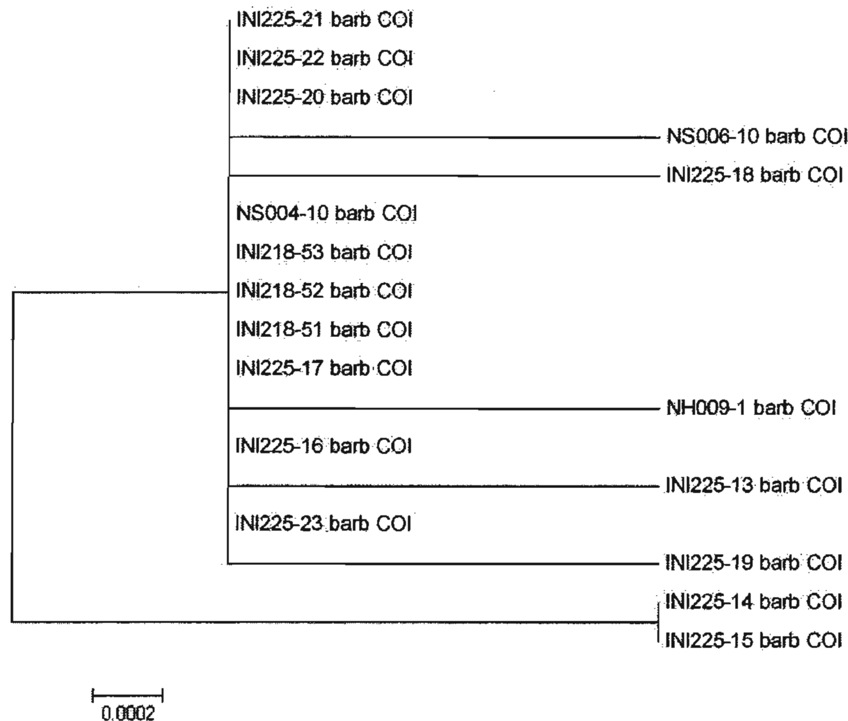

Figure 3.3. Neighbour-joining analysis of K2P distances of *An. barberi* COI sequences. Note: collection locations (NI, NH, and NS) are all from the same woodlot in Niagara.

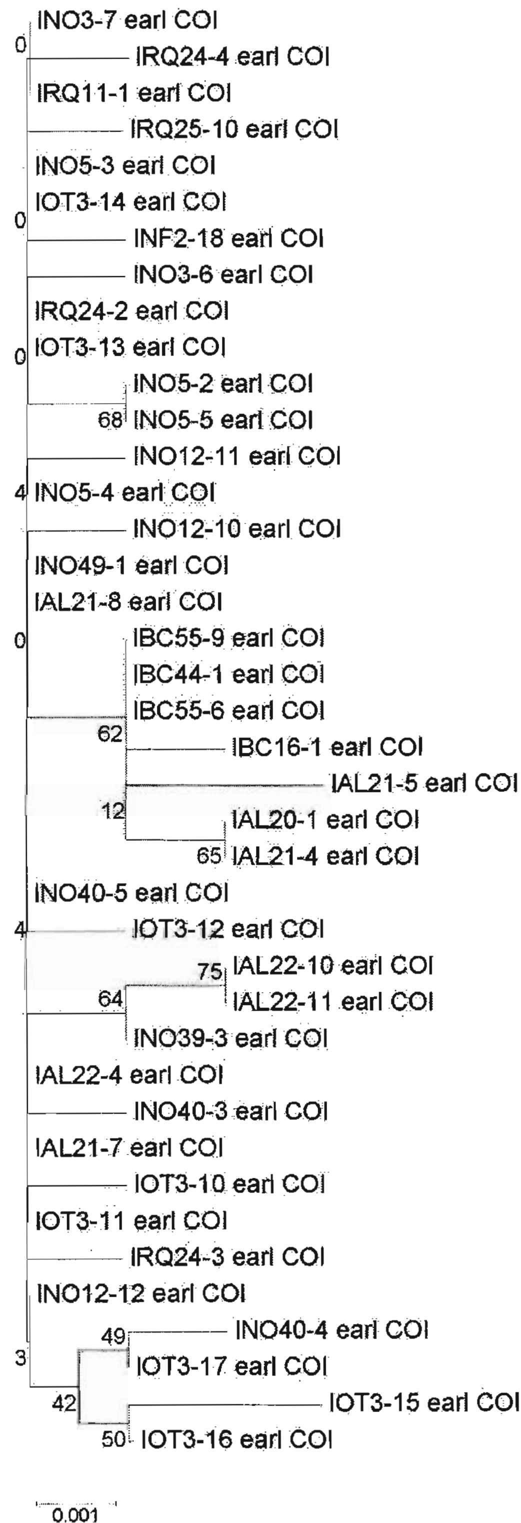

Figure 3.4. Neighbour-joining analysis of K2P distances of *An. earlei* COI sequences. Region codes are listed in Appendix I.

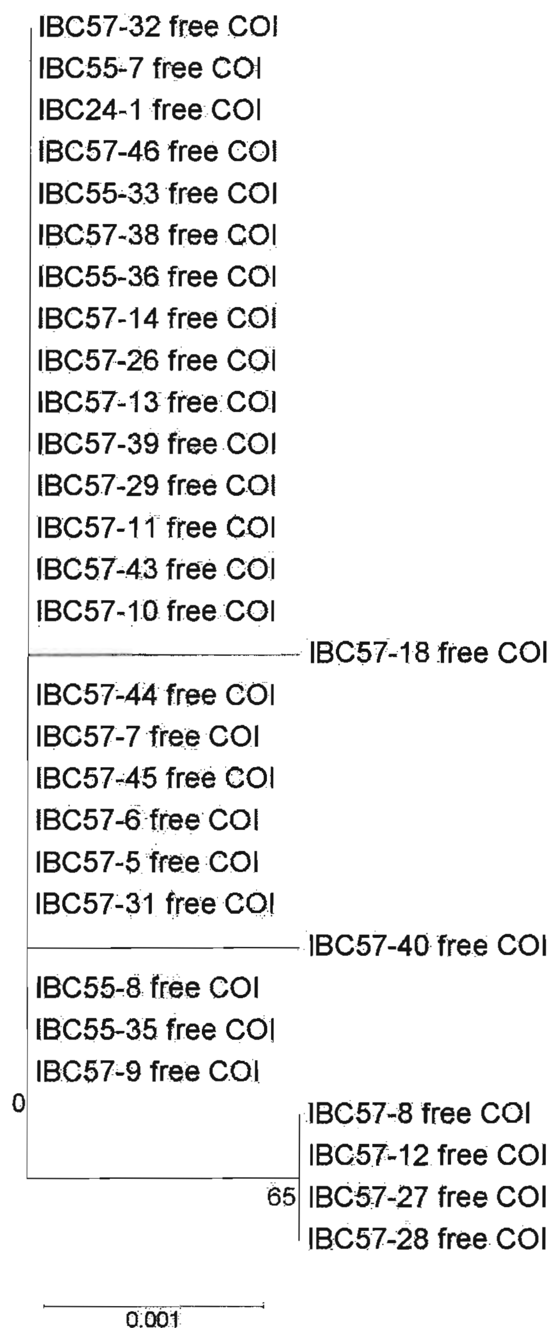

Figure 3.5. Neighbour-joining analysis of K2P distances of *An. freeborni* COI sequences. Region codes are listed in Appendix I.

Anopheles punctipennis and *An. perplexens* COI

COI sequences were obtained from 79 *An. punctipennis*- and *An. perplexens*-type larvae and adults collected from a wide variety of locations across Canada, with 32 specimens identified morphologically as *An. perplexens* and 47 as *An. punctipennis* (Table 3.3). K2P sequence divergences were relatively high, ranging from 0-2.7% with a mean of 0.9%, with 57 haplotypes and 70 variable sites present among the 79 sequences (Table 3.5). NJ analysis of K2P distances revealed two distinct clusters within *An. punctipennis* and *An. perplexens* samples: a) those collected from BC, and b) those from various regions throughout Ontario, including WE, NI, HL, NO, AL and BP (Figure 3.6). Little variation was present within *An. punctipennis* samples from British Columbia (three haplotypes among 14 specimens) compared to those from Ontario (53 haplotypes among 67 specimens). Sequence divergence levels were significantly greater between the two groups, from 1.7-2.6%, compared to within them; pairwise sequence divergences within BC specimens ranged from 0-0.4%, and from 0-1.2 % within those from ON. Within the ON group, distinct clusters of specimens based on either morphological identification (*An. perplexens* or *An. punctipennis*) or region of collection (WE, NI, HL, NO, AL and BP) were not present (Figure 3.6).

Anopheles quadrimaculatus s.l. COI

COI sequences were obtained from 60 *An. quadrimaculatus* and *An. smaragdinus* larvae and adults collected from various regions in Ontario (NI, WE, and OT), with 52 specimens identified morphologically as *An. quadrimaculatus* and 8 as *An. smaragdinus* (Table 3.3). There were 46 haplotypes, and 52/806 variable sites, among the 60 COI sequences, which had K2P sequence divergences ranging from 0-1.2% with an average sequence divergence of 0.6% (Table 3.5). NJ analysis of all *An. quadrimaculatus s.l.* and *An. smaragdinus* COI sequences did not reveal any distinct clusters based on morphological identification or collection location (Figure 3.7).

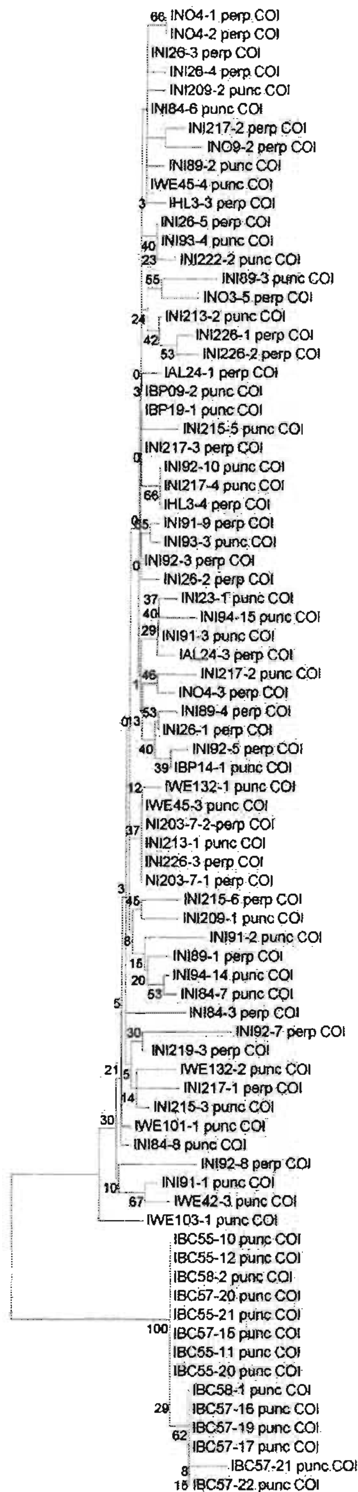

Figure 3.6. Neighbour-joining analysis of K2P distances of COI sequences from *An. punctipennis* (punc) and *An. perplexens* (perp) specimens. Region codes listed in Appendix I.

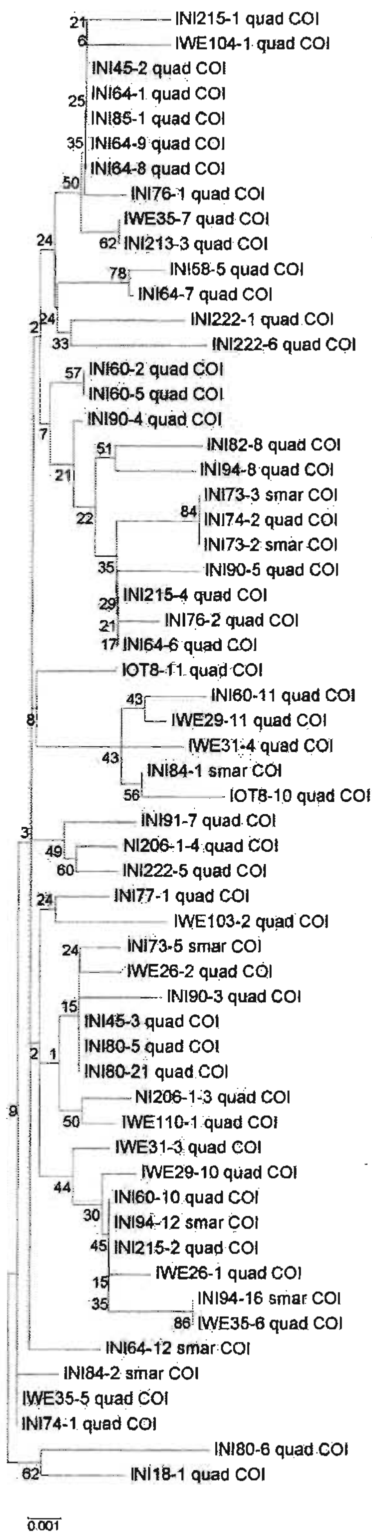

Figure 3.7. Neighbour-joining analysis of K2P distances of *An. quadrimaculatus s.l.* and *An. smaragdinus* COI sequences. Collection locations include; NI – Niagara, WE – Windsor/Essex County, OT – Ottawa.

Anopheles walkeri COI

COI sequences were obtained from 12 *An. walkeri* adults collected from two regions in southern Ontario; six from LP and six from NO (Table 3.3). Intraspecific variation was highest in this species, with K2P sequence divergences ranging from 0-8.2% with an average of 4.6% (Table 3.5). Sequence divergence was lower within each geographic group than between them, ranging from 0.4-3.1% within LP specimens and from 0-2.0% within those from NO. Between the two groups, sequence divergences ranged from 6.7-8.2% (Figure 3.8).

Combined COI analysis of all species

In addition to intraspecific analyses of COI sequences for each species included in the study, interspecific differences were analysed as well. Interspecific variation was significantly greater than intraspecific variation (ranged from 0 to ~2.0% in most species, but was higher, 3.1%, within the LP *An. walkeri* group). Sequence divergence between species ranged from 6.7% (*An. freeborni* and *An. punctipennis*) to 13.9% (*An. barberi* and *An. quadrimaculatus s.l.*) (Table 3.6). Within the anopheline species, sequence divergence levels were highest in *An. barberi*, ranging from 11.4-13.9% between *An. barberi* and the remaining species. Sequence divergence levels between *An. barberi* and the other (marsh) species were almost as high as those between *Culex territans* and all anophelines included in the current study (i.e., 14.5-16.3%) (Table 3.6). Excluding *An. barberi* (the only non-marsh species included in the current study), sequence divergence levels were lowest among *An. earlei*, *An. freeborni*, and *An. punctipennis* (including specimens identified as *An. perplexens* and those from BC that likely represent a cryptic species within this taxon), ranging from 6.7-7.2%. The next highest sequence divergence levels were seen between *An. quadrimaculatus s.l.* (including specimens identified as *An. smaragdinus*) and the other species, ranging from 8.2% (*An. punctipennis*) to 12.3% (*An. walkeri*). *An. walkeri* (including specimens from LP and NO that likely represent cryptic species within this taxon) exhibited the highest levels of sequence divergence, from 10.4% (*An. punctipennis*) to 12.3% (*An. quadrimaculatus s.l.*) (Table 3.6).

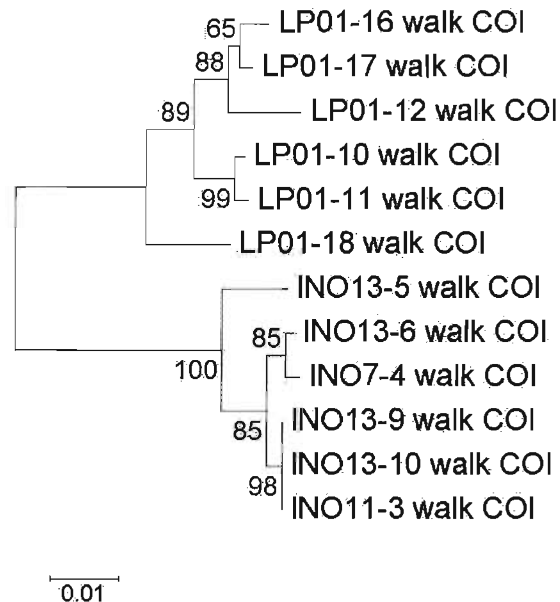

Figure 3.8. Neighbour-joining analysis of K2P distances of COI sequences from *An. walkeri* specimens. Collection locations include; LP - Long Point Provincial Park, NO - Manitoulin Island.

Table 3.6. Kimura two-parameter (K2P) COI sequence divergence levels between each known *Anopheles* species examined, and one *Culex* species, *Cx. territans* (as an outgroup).

	B	E	F	P	Q	W
An. barberi						
An. earlei	0.121					
An. freeborni	0.128	0.072				
An. punctipennis/perplexens	0.114	0.070	0.067			
An. quadrimaculatus/smaragdinus	0.139	0.094	0.100	0.082		
An. walkeri	0.134	0.108	0.107	0.104	0.123	
Cx. territans	0.163	0.162	0.145	0.145	0.149	0.159

Neighbour-joining analysis of all unique COI haplotypes obtained revealed eight distinct anopheline groups (branch support 80% or higher): *An. barberi, An. earlei, An. freeborni, An. punctipennis* (BC group), *An. punctipennis* (ON group, including specimens identified as *An. perplexens*), *An. quadrimaculatus s.s.* (including specimens identified as *An. smaragdinus*), *An. walkeri* (NO group), and *An. walkeri* (LP group) (Figure 3.9). Similar to the results of Porter and Collin (1996), *An. earlei* and *An. freeborni* form sister taxa, which are closely related to *An. punctipennis* (BC and ON groups). Next is *An. quadrimaculatus,* followed by *An. walkeri* (NO and LP groups). The species with the most divergent COI sequences was *An. barberi*, the tree hole specialist that was most distinct in each of the types of data analysed.

3.3.2. ITS2

A total of 254 ITS2 sequences were obtained for analysis (Table 3.7). Since ITS2 rDNA is not protein coding, insertions and deletions (indels) are free to accumulate, and processes such as concerted evolution usually ensure that mutations in one rDNA subunit spread to all of the tandemly repeated rDNA units quickly, resulting in uniformity of subunits within the rDNA array. Therefore, the lengths of the ITS2 fragments were varied and species-specific. ITS2 PCR products of all species examined were single bands ranging in size from ~400-800bp in length (Figure 3.10).

Total length of the ITS2 fragments examined (excluding *An. earlei*) ranged from 374bp (*An. punctipennis*) to 422bp (*An. freeborni*), which contained 77bp of 5.8S rDNA at the 5′ end and 27bp of 28S rDNA on the 3′ end. Thus, the length of the ITS2 sequence only (i.e., 5.8S and 18S rDNA nucleotides removed) in most species ranged from 270bp (*An. punctipennis*) to 318bp (*An. freeborni*) (Table 3.8), but was difficult to determine exactly in *An. earlei* due to difficulty sequencing the last ~450bp of the ITS2 sequence. All *An. earlei* ITS2R sequences (n=41) resulted in clear signals for the first ~200bp when a second signal becomes very strong, resulting in a large number of questionable base calls that can't be resolved using the forward sequence. Therefore, only the first 318bp of the ITS2 sequence of *An. earlei* was included in the following analyses.

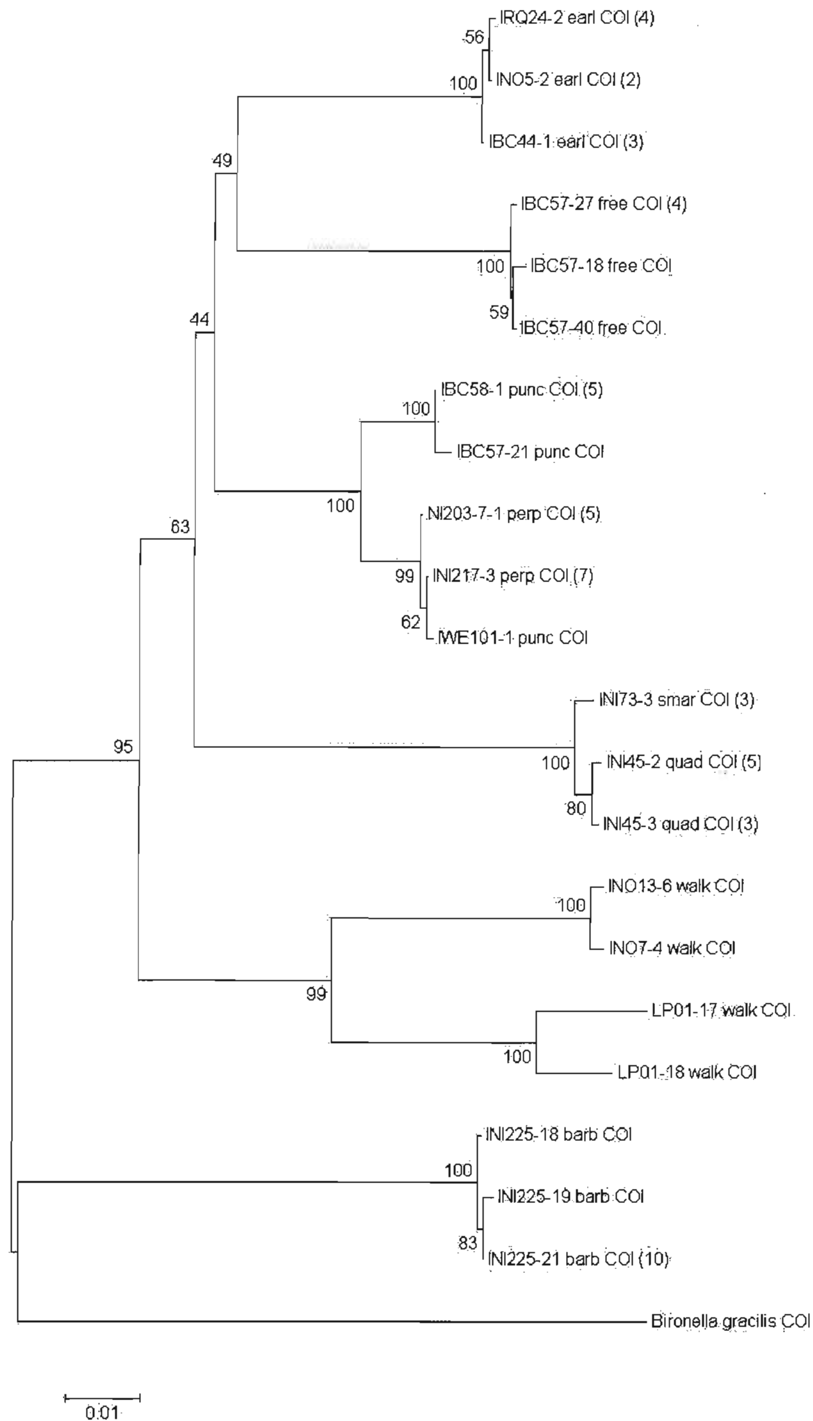

Figure 3.9. Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of representative COI sequences from *Anopheles* mosquitoes collected in Canada and one *Bironella gracilis* (outgroup species). When multiple sequences shared the same haplotype, the number of sequences that shared each one is included in parentheses. Region codes are listed in Appendix 1.

Table 3.7. Number of individuals of each species from each region for which ITS2 sequences were obtained. Region codes listed in Appendix I. Note: "*An. smaragdinus*" includes two specimens identified morphologically as other members of *An. quadrimaculatus* complex; one *An. inundatus* and one *An. diluvialus*.

	BC	NI	WE	NO	OT	LP	NF	RQ	AL	HL	BP	**Total**
An. barberi		18										**18**
An. earlei	5			15	7		1	5	8		1	**41**
An. freeborni	26											**26**
An. perplexens		25		5	3				2	2		**34**
An. punctipennis	11	25	10								3	**49**
An. quadrimaculatus s.l.		42	12		2							**56**
An. smaragdinus		16										**16**
An. walkeri				5		5						**10**
Total	42	126	22	25	12	5	1	5	10	2	4	**254**

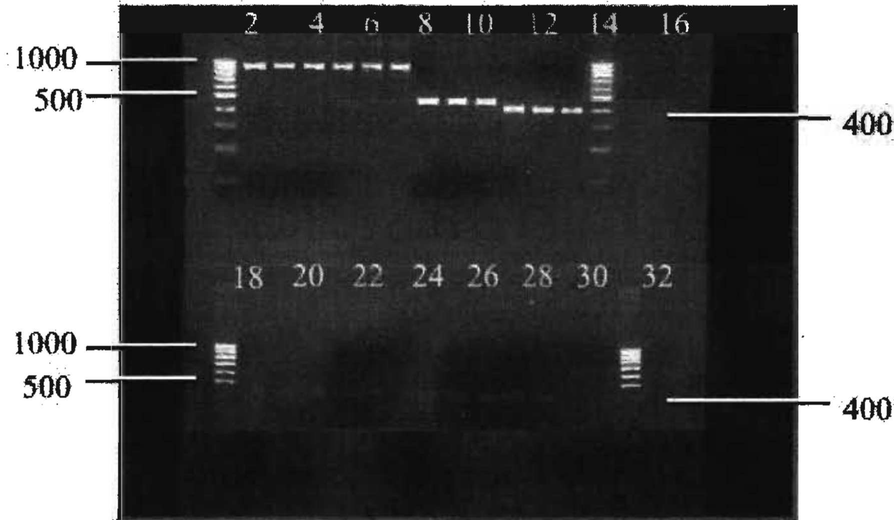

Figure 3.10. ITS2 PCR products for five *Anopheles* species. Lanes 1, 14, 18, and 31 are DNA band size ladders, labeled bands in base pairs (bp). Lanes 2-7 = *An. earlei,* lanes 8-10 = *An. freeborni*, lanes 11-13 = *An. punctipennis*, lanes 18-23 = *An. walkeri,* and lanes 24-28 = *An. barberi.*

Sequencing in the reverse direction was also difficult in *An. walkeri* (but not noticed in time to design a species-specific reverse primer), but only in the specimens from LP. Therefore, in *An. walkeri* from NO, the ITS2 (including 5.8S and 28S sequences) was 406bp in length, with the ITS2 sequence only (i.e., 5.8S and 28S removed) 302bp in length (Table 3.8). However, in the *An. walkeri* from LP, sequencing in the forward direction results in a strong, clear signal until ~275bp when a second signal appears. At ~325bp, the second signal starts to interfere with sequencing and it becomes weak and completely unreadable by ~375bp. All of the ITS2 reverse reactions in LP specimens were unsuccessful. Therefore, the ITS2 sequences from NO and LP *An. walkeri* specimens were reduced to the first 275bp in the forward direction (Tables 3.8 and 3.9)

The ITS2 sequences of all species examined had similar GC contents ranging from 50.7% in *An. barberi* to 57.2% in *An. walkeri*, with those of *An. earlei*, *An. freeborni*, and *An. punctipennis* (including those identified as *An. perplexens*) 51.5-52.8%, and *An. quadrimaculatus*, 54.0% (Table 3.8). Four species exhibited little to no intraspecific variation within ITS2 (*An. barberi*, *An. earlei*, *An. freeborni*, and *An. quadrimaculatus s.l.*), but significant intraspecific variation was observed in the other two (i.e., *An. punctipennis* and *An. walkeri*) (Table 3.9).

All *An. freeborni* specimens (n=32) had identical ITS2 sequences, as did all of the *An. quadrimaculatus s.l.* specimens (n=73), including those identified as *An. smaragdinus* and other members of the *An. quadrimaculatus* complex (n=16). The *An. barberi* sequences (n=18) contained one specimen with a single nucleotide change. Intraspecific variation was higher within the *An. earlei* sequences (n=41), with two variable sites within the 318bp *An. earlei* ITS2 sequences that resulted in two distinct groups, those from BC (n=5) in one group and those from all other locations, including ON, RQ and NF, in the second (Figure 3.11). The ITS2 sequences of the remaining species, however, revealed significant intraspecific variation.

The *An. punctipennis* and *An. perplexens* ITS2 sequences resulted two distinct groups, with all *An. punctipennis* from BC in one group (n=11) and those from locations throughout ON in a second group (n=75), which included all specimens identified as *An. perplexens* (Figure 3.12). ITS2 sequences within each group were identical, but sequence

Table 3.8. Summary statistics of ITS2 sequences from all *Anopheles* species examined. Included are average base compositions over all codons, including combined GC content, and both the total number of nucleotides (nts) included in each ITS2 sequence analysed (includes portions of 5.8S and 28S) and the number of nucleotides of the ITS2 region only, for each species examined. [A] reduced sequence (5′ end, 5.8S + partial ITS2). [B] only NO specimens since those from LP did not sequence well.

	T	C	A	G	GC%	Total nts	ITS2 nts
An. barberi	24.8	26.4	24.5	24.3	50.7	379	275
An. earlei [A]	21.4	24.8	27.1	26.7	51.5	318[A]	299[A]
An. freeborni	22.3	27.7	24.9	25.1	52.8	422	318
An. perplexens	21.3	27.7	26.1	24.8	52.5	375	271
An. punctipennis	21.2	27.6	26.0	25.1	52.7	374	270
An. quadrimaculatus s.l.	21.0	28.9	25.1	25.1	54.0	395	291
An. smaragdinus	21.0	28.9	25.1	25.1	54.0	395	291
An. walkeri [B]	19.0	29.6	23.9	27.6	57.2	406 [B]	302 [B]

Table 3.9. p-Distance sequence divergence levels and number of unique sequences and variable sites among ITS2 sequences for all species examined.

	n	Min	Max	Number Unique Sequences	Number Variable Sites
An. barberi	18	0.000	0.004	2	1/275
An. earlei (318bp)	41	0.000	0.006	2	2/318
An. freeborni	26	0.000	0.000	1	0/422
An. punctipennis/perplexens	86	0.000	0.091	2	34/375
An. quadrimaculatus/smaragdinus	73	0.000	0.000	1	0/395
An. walkeri (231bp)	11	0.000	0.074	2	17/231

```
lBC55-6_earl_ITS2_350       ACACATTTTTGAGTGCCCATATTTGACTAATCCAAGTCAAACTACGCCGG 50
lNF2-18_earl_ITS2_350       ACACATTTTTGAGTGCCCATATTTGACTAATCCAAGTCAAACTACGCCGG 50
                            **************************************************

lBC55-6_earl_ITS2_350       CGAGGCCAGCCCTTGCCGTGCGTGCATAGATGATGAAAGAGTATGGGACC 100
lNF2-18_earl_ITS2_350       CGAGGCCAGCCCTTGCCGTGCGTGCATAGATGATGAAAGAGTATGGGACC 100
                            **************************************************

lBC55-6_earl_ITS2_350       TAAACCATCCCATTTCTTGCATTGAAAGCGAAGCGTGTAATCCAGGGAGT 150
lNF2-18_earl_ITS2_350       TAAACCATCCCATTTCTTGCATTGAAAGCGAAGCGTGTAACCCTGGGAGT 150
                            **************************************** ** ******

lBC55-6_earl_ITS2_350       TCACTTGCAAAGTGGCCCTTGGCCAACACCTCACCACCAACGGCGGTGCT 200
lNF2-18_earl_ITS2_350       TCACTTGCAAAGTGGCCCTTGGCCAACACCTCACCACCAACGGCGGTGCT 200
                            **************************************************

lBC55-6_earl_ITS2_350       GTGCAGTGTGTTTGGCTGAGTACGGACCATCGTGAGTTGGACTCCCAACC 250
lNF2-18_earl_ITS2_350       GTGCAGTGTGTTTGGCTGAGTACGGACCATCGTGAGTTGGACTCCCAACC 250
                            **************************************************

lBC55-6_earl_ITS2_350       GTATCTCGTGGTGGACACAGTGGACAGGGAGTCCACTATAAACACAAAGG 300
lNF2-18_earl_ITS2_350       GTATCTCGTGGTGGACACAGTGGACAGGGAGTCCACTATAAACACAAAGG 300
                            **************************************************

lBC55-6_earl_ITS2_350       TCAAGAGAGAGAGAGAGA 318
lNF2-18_earl_ITS2_350       TCAAGAGAGAGAGAGAGA 318
                            ******************
```

Figure 3.11. Clustal alignment of *An. earlei* ITS2 sequences (318bp). Variable sites are in bold. The lBC55-6 sequence represents the BC group (n=5) and the lNF2-18 sequence represents the second group, which contains specimens from all other regions (ON, RQ, and NF) (n=26).

```
lBC57-17_punc_ITS2     ACACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTAC 60
lWE103-1_punc_ITS2     ACACATGAACACCGATAAGTTGAACGCATATTGCGCATCGTGCGACACAGCTCGATGTAC 60
                       ************************************************************

lBC57-17_punc_ITS2     ACATTTTTGAGTGCCCATATTTGACCCTCCAAGTCAAACTACGCCGGCCTGGCCGTGCGT 120
lWE103-1_punc_ITS2     ACATTTTTGAGTGCCCATATTTGACCCTCCAAGTCAAACTACGCCGGCCTAGCCGTGCGT 120
                       ************************************************** *********

lBC57-17_punc_ITS2     GCAGTATGATGTCACTGGTGGTGGCATTGAAAACGTAGCGTGCACCCTAGGGCTCAACTT 180
lWE103-1_punc_ITS2     GCATAATGATGTCACTGGTTGTGGCATTGAAAACGATGCGTGCACCCCAGGGCTCAACTT 180
                       ***  ************** ***************  ********** ************

lBC57-17_punc_ITS2     ACAGAGTGACCACGGGGCCGACAGCTCACCAAAGTAACCTAATACGTTGAAGCGTGCAGA 240
lWE103-1_punc_ITS2     ACAGATTGGACTCGGGACCGACAGCTCACCAAAGTAACCTAATACGTTGAAGCGTGCAAG 240
                       ***** **  * **** *****************************************

lBC57-17_punc_ITS2     -CTGTTCGGCCCGGTTCGGTCATCGTGAGGCGAGTCCGTGGTGCACACACACAA--CGAG 297
lWE103-1_punc_ITS2     TCTGTTCGGCCCGGT-CAGTCATCGTGAGGCGAGTCCATCGTGCACGCACACACTTCGAG 299
                        ************** * ******************* * ****** ******   ****

lBC57-17_punc_ITS2     TCGCATGGTTGCGAACAAG---ACATGCTCCTAGCAGCGGGAGTACATGGGCCTCAAATA 354
lWE103-1_punc_ITS2     TCGTGTGACAGCGACCGAAGAAACATGCTCCTAGCAGCGGGAGTACATGGGCCTCAAATA 359
                       ***  **   **** * *    **************************************

lBC57-17_punc_ITS2     ATGTGTGACTACCCCC 370
lWE103-1_punc_ITS2     ATGTGTGACTACCCCC 375
                       ****************
```

Figure 3.12. Clustal alignment of BC and ON *An. punctipennis* ITS2 sequences (370bp and 375bp, respectively). Variable sites are in bold. lBC57-17 represents the BC group (n=11) and lWE103-1 the ON group (n=75).

divergence between the two groups was 9.1%, with 27 variable sites within the 375bp sequence (Table 3.9; Figure 3.12).

Similarly, the *An. walkeri* ITS2 sequences of specimens from NO and LP were identical within each group, but very different between groups. There were 17 variable sites within the 231bp sequence, a sequence divergence of 7.4% between the two groups (Table 3.9; Figure 3.13). Therefore, since intraspecific variation in the ITS2 sequences of *An. punctipennis* (9.1%) and *An. walkeri* (7.4%) is within that known for members of other anopheline species complexes, which range from 0.4-1.6% in the *An. gambiae* complex (Paskewitz *et al.* 1993) to 18.5-28.7% in the *An. quadrimaculatus* complex, these data provide further evidence for the presence of cryptic species within these taxa.

ITS2 combined analysis of all species

The ITS2 sequences of the anophelines examined varied in length depending on species and therefore could not be analysed using MEGA 4.0 for interspecific differences. Clustal2W was used to align the sequences and generate a Neighbour-joining tree (Figure 3.14), which resulted in similar relationships among species as that based on COI sequence (Figure 3.9), except for *An. earlei*, which is likely due to the much larger size of the PCR product (~800bp) and the requirement for editing of the final sequence.

3.3.3. ITS1

The number of ITS1 sequences obtained for analysis was much lower than for COI and ITS2. This was due to the inability of the primers to amplify ITS1 in all species examined and to successfully sequence the ITS1 PCR products, likely the result of intragenomic variation. The ITS1 primers designed for this study (Table 3.2) amplified the ITS1 region successfully in all species, except *An. barberi*. ITS1 PCR products ranged in size from ~600bp in *An. walkeri* to ~4500bp in *An. freeborni* (Figure 3.15). Both *An. quadrimaculatus s.l.* and *An. walkeri* resulted in single bands at ~600bp and

```
1NO7-4_walk_ITS2_229bp      ACACATTTTTGAGCGCCCATATTTGACCATATCAATCAAACTATGCCGCG 50
LP01-10_walk_ITS2_231bp     ACACATTTTTGAGCGCCCATATTTGACCATATCAATCAAACTATGCCGCG 50
                            **************************************************

1NO7-4_walk_ITS2_229bp      CACGGCCGAAAGGCCACGCCGGCGTGCAACGCGCCCACCC--GGGCTGCT 98
LP01-10_walk_ITS2_231bp     TGCGACCGAACGGCCGCGCCGGTGTGCAACGCGCCCGCCCCAGGGCTGCT 100
                              ** ***** **** ****** ************* ***  ********

1NO7-4_walk_ITS2_229bp      AACTGATGGAGGGGATCGGCAGCGTCCGGAGTCCTTTCATTGAAATCTAC 148
LP01-10_walk_ITS2_231bp     AACTGATGGAGGGGATCGGCAGCGTCCGGAGTCCTTTCATTGAAACCTAC 150
                            ********************************************* ****

1NO7-4_walk_ITS2_229bp      CGCTGAGTGTTTTCTCCCGGGCTGCACCCAGTCGGGACACCACCCTGTGG 198
LP01-10_walk_ITS2_231bp     CGCTGAGTGTATTCTCCCGGGCTGCGCCCAGTCGGGACACCACCCTGTGG 200
                            ********** ************** ************************

1NO7-4_walk_ITS2_229bp      ATGAGACACCTCACCAGCTTTGCAACGGGCA 229
LP01-10_walk_ITS2_231bp     ATGAGACACCTCACCAGCTTTGCAACAACGG 231
                            **************************
```

Figure 3.13. Clustal alignment of edited NO and LP *An. walkeri* ITS2 sequences (229bp and 231bp, respectively). Variable sites are in bold. The 1NO7-4 sequence represents the NO group (n=5) and the LP01-10 the LP group (n=6).

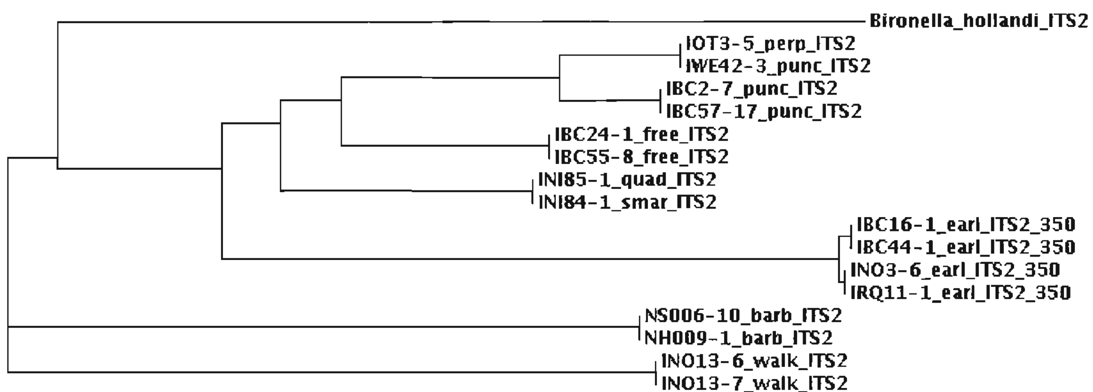

Figure 3.14. Neighbour-joining tree generated using Clustal2W based on representative ITS2 sequence of all species examined. Specimen codes indicate collection location (listed in Appendix I) and species identification based on morphological data.

~800bp, respectively, allowing the PCR products to be easily sequenced in both the forward and reverse directions. *An. freeborni* and *An. earlei* also resulted in single bands, but PCR products were considerably larger, approximately 4500bp and 4000bp, respectively, preventing sequencing of the entire fragments in both directions. Some *An. freeborni* and *An. earlei* specimens resulted in ~750bp of ITS1 sequence in each direction, resulting in ~1500bp of ITS1 sequences for analysis (Figure 3.16).

The ITS1 region of *An. punctipennis*, however, was more complicated. PCR resulted in multiple bands in almost all specimens examined, except those from BC. Although band sizes varied, most *An. punctipennis* specimens resulted in three bands at ~1250bp, ~2500bp, and ~3000bp (Figure 3.15). Because PCR products could not be sequenced directly, very few *An. punctipennis* ITS1 sequences were obtained for analysis. Detailed descriptions of the ITS1 sequences of all species for which they were obtained are provided below.

Anopheles earlei ITS1

PCR amplification of ITS1 in *An. earlei* resulted in single, large bands at ~4000bp (Figure 3.15), but sequencing of ITS1 PCR products for the first ~750bp in each direction resulted in sequences of poor quality. Ten ~750bp ITS1F sequences were obtained, but three were excluded from further analysis due to poor quality. Of the seven remaining ~750bp ITS1F sequences, all were identical for the first ~350bp, after which another weaker signal appeared, with questionable bases increasing in frequency for the next ~300 base pairs, and the sequence almost completely unreadable around the 670th base pair. Sequencing in the reverse direction was similar but even less successful. Ten ~750bp ITS1R sequences were obtained, but seven were excluded from further analysis due to poor quality. Of the three ~750bp ITS1R sequences, all were almost identical for the first ~500bps, after which sequence quality began to quickly deteriorate.

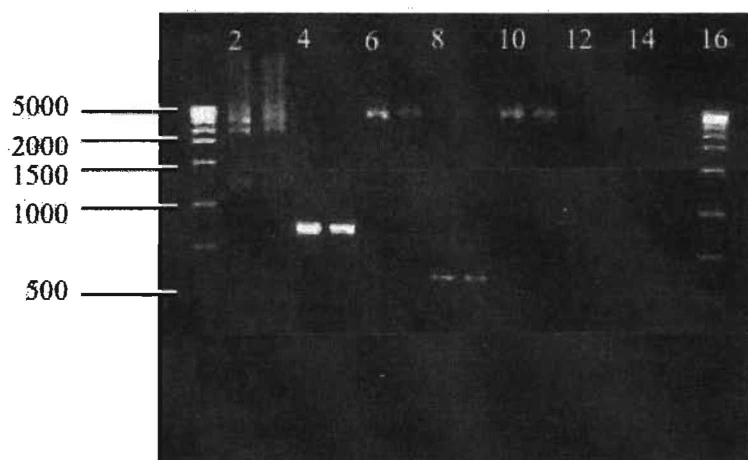

Figure 3.15. ITS1 PCR products of all six *Anopheles* species examined. Lanes: 1, 16 = DNA ladder; 2, 3 = *An. punctipennis* (3000bp, 2500bp, faint 1250bp); 4, 5 = *An. quadrimaculatus* (800bp); 6, 7 = *An. earlei* (4000bp); 8, 9 = *An. walkeri* (600bp); 10, 11 = *An. freeborni* (4500bp); 12, 13 = *An. barberi* (no bands); 14, 15 = negative controls.

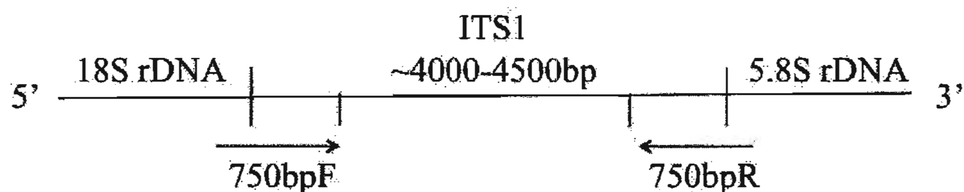

Figure 3.16. ITS1F and ITS1R regions sequenced in *An. earlei* and *An. freeborni*. ITS1 PCR products were ~4000bp and ~4500bp, respectively, and forward and reverse sequencing reactions resulted in ~750bp of sequence in each direction beginning near the 5′ and 3′ ends of the ITS1 region.

Anopheles freeborni ITS1

The largest ITS1 PCR product obtained was that of *An. freeborni,* which was a single band at ~4500bp, similar to that of *An. earlei* (Figure 3.15). However, sequencing of the first ~750bp in each direction resulted in much better quality sequences than those of *An. earlei*. All nine 806bp *An. freeborni* ITS1F sequences were identical for the first ~650bp, and only 9 variable sites were found in the next ~150bp. In the reverse direction, all nine 751bp ITS1R sequences were identical throughout most of the sequence, with only 2 variable sites located at ~240 and ~480 base pairs into the sequence. Therefore, although there were some nucleotide differences among the nine *An. freeborni* specimens, there was no evidence of intragenomic variation in this species within the ~1500bp of sequence examined.

Anopheles punctipennis ITS1

PCR amplification of the ITS1 region in *An. punctipennis* produced multiple bands in almost all individuals examined, the three most common of which were ~1250bp, ~2500bp, and 3000bp in length (Figure 3.15). This suggests the potential presence of intraindividual variation within ITS1 in *An. punctipennis*, i.e., different ITS1 sequences within the rDNA array. The number, size, and intensity of ITS1 bands were highly variable in *An. punctipennis* specimens (Figure 3.17). A smear was often observed from the well until the appearance of the first two bands at ~2500 and ~3000bp (Figure 3.17, lanes 15, 16, 19, 21, etc.), and sometimes additional, smaller bands were produced (Figure 3.17, lanes 5 and 12). The intensity of each particular band also varied, with some individuals having the largest band the brightest or most intense (Figure 3.17, lanes 24 and 25), and others with the smallest band the brightest (Figure 3.17, lanes 3 and 11).

Within the *An. punctipennis* specimens from ON, there were no apparent patterns in the number, size, or intensity of bands to suggest population-specific differences (Table 3.10). Most common were the two largest bands (~2500bp and ~3000bp), which

were present in all ON specimens (n=46) (i.e. from AL, BP, NO, HL, NI, and WE). The ~1250bp band was present in all samples (n=11) from more northern sites (AL, BP, and NO), as well as those from WE (n=8) and HL (n=2), but was only present in a quarter (8/32) of those from NI. The next most common band (~2000bp) was present in 15/46 ON specimens, and most often in those identified morphologically as *An. perplexens* from NO (5/7). The remaining bands that were observed in *An. punctipennis* ITS1 samples were ~1700bp, 1500bp, 950bp, 825bp, 650bp, and 450bp in length, and occurred rarely (i.e., in one to four of 46 specimens). In cases where ITS1 was sequenced in more than one specimen from a single collection site (Table 3.10, e.g., lNO4, lHL3, lNI209, lNI213, etc.), more than one band pattern was usually present among the individuals from that site.

There was, however, one geographic difference observed among *An. punctipennis* ITS1 sequences. All specimens collected from BC (n=4) produced single bands (Figure 3.18). Although they varied in size considerably (Table 3.10), they were the only specimens (4/50) to produce only one band. With respect to morphological identification, the only obvious difference between those identified as either *An. perplexens* or *An. punctipennis* was the higher proportion of specimens with the ~2000bp band in *An. perplexens* (12/21 or 57%) compared to *An. punctipennis* (4/30 or 13%). Because sequencing of PCR products with multiple bands was not possible, ITS1 sequences were not obtained for *An. punctipennis*.

***Anopheles quadrimaculatus s.l.* ITS1**

Despite sequencing a large number of *An. quadrimaculatus s.l.*, including those identified morphologically as *An. smaragdinus*, COI and ITS2 data did not reveal evidence of cryptic species in this taxon. However, the ITS1 sequences of *An. quadrimaculatus s.l.* (n=45) formed two distinct groups. Both groups produced single ITS1 bands at ~800bp, but after consensus sequence determination, the length of the ITS1 sequences differed by ~25 nucleotides between groups (Figure 3.15). The ITS1 sequence of the first group was 729bp in length, and contained NI, WE, and OT specimens (n=35), and was 756bp in length in the second group, with specimens from NI and WE (n=10). Within each group, the sequences were identical, but there were major differences

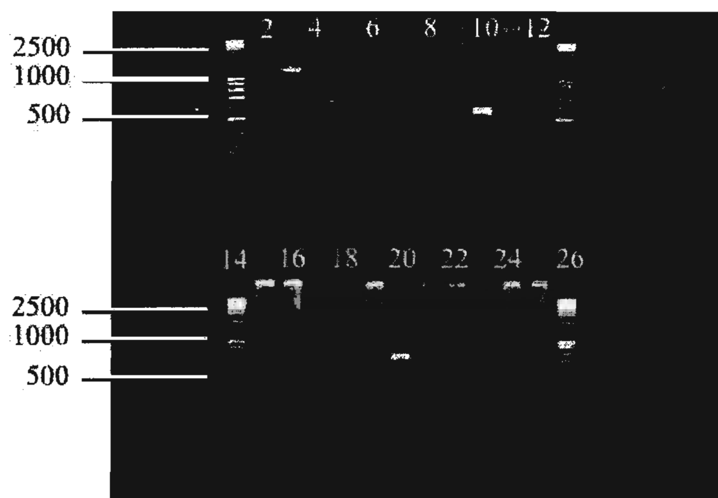

Figure 3.17. ITS1 PCR products from 21 *An. punctipennis* specimens. Lanes: 1, 13, 14, 26 = DNA ladder; 10, 20 = *An. quadrimaculatus s.l.*; 2-5 = WE punc; 6-8 = BP punc; 9, 11, 12, 15-19, 22 = NI punc; 21, 24, 25 = NI perp.

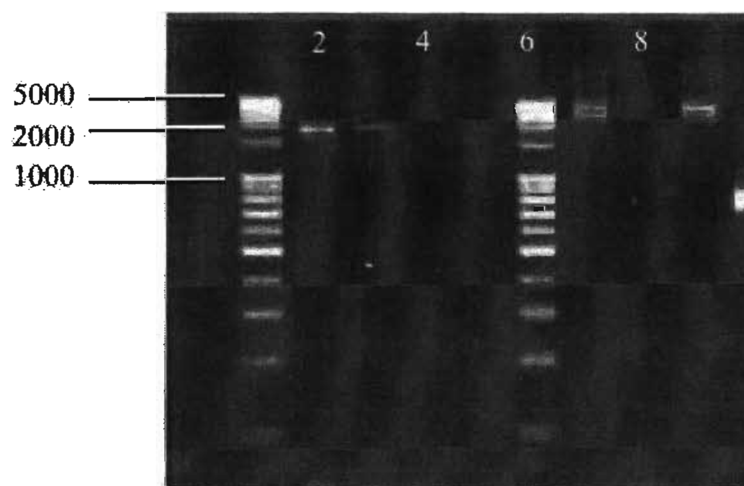

Figure 3.18. ITS1 PCR products BC and NI *An. punctipennis* specimens. Lanes: 1, 6 = DNA ladders, 2-5 = BC *An. punctipennis*, 7-9 = Niagara *An. punctipennis*.

Table 3.10. Size and intensity of ITS1 bands from specimens identified morphologically as of *An. punctipennis* and *An. perplexens*. Abbreviations: vb – very bright; b – bright; m – medium; f – faint; vf – very faint.

Code	Species	3000	2500	2000	1700	1500	1250	950	825	650	450
1BC57-20	punc				m						
1BC57-21	punc				m						
1BC57-41	punc			vb							
1BC57-42	punc						f				
1AL24-3	perp	f	f				vf				
1BP09-2	punc	m	m				f				
1BP14-1	punc	f	f				vf				
1BP19-1	punc	f	f				f				
1NO3-5	perp	b	vb				f				
1NO4-1	perp	b	b	f			m				
1NO4-2	perp	b	b	f		f	m				
1NO4-3	perp	f	f	vf			vf				
1NO4-4	perp	b	b	f			m				
1NO4-5	perp	b	b				f		b		
1NO9-2	perp	b	b	f			vf				
1HL3-3	perp	vf	vf				f				
1HL3-4	perp	b	b	f		m	f				
1NI209-1	punc	vf	vf								
1NI209-2	punc	vf	vf				vf				
1NI213-1	punc	vb	b	f			m				
1NI213-2	punc	b	vb				m				
1NI215-3	punc	b	m				f				
1NI215-5	punc	b	m								
1NI215-6	perp	vb	vb	vf							
1NI217-1	perp	m	m								
1NI217-2	perp	f	f			vf	vf				
1NI217-3	perp	vb	vb	f			b				
1NI217-4	punc	b	b								
1NI219-3	perp	vb	vb	m	vf						
1NI222-2	punc	b	b								
1NI222-4	pun	b	b				vb				
1NI226-5	punc	b	b				f			f	
1NI26-1	perp	vb	b								
1NI26-2	perp	f	vf								
1NI26-3	perp	b	b	m							
1NI26-4	perp	b	m								
1NI84-6	punc	m	m								
1NI84-8	punc	b	m								
NI203-7-1	perp	vb	m	f							
NI203-7-2	perp	vb	f	f							
NI216-1-3	punc	f	f								
NI216-1-4	punc	f	f								
1WE101-1	punc	f	f				vb				
1WE103-1	punc	m	m				f				m
1WE132-1	punc	m	m				f				
1WE132-2	punc	f	f				f				
1WE42-3	punc	b	b		vf	f	b				
1WE44-4	punc	f	f				f				
1WE45-2	punc	b	b	vf			b				
1WE45-3	punc	b	b	vf			b	m			

between the two groups, spread evenly throughout the entire sequence (Figure 3.19). There was an intraspecific sequence divergence of 55.7% between the two groups, the highest level of variation observed among all species and markers examined.

Anopheles walkeri ITS1

PCR amplification of ITS1 in *An. walkeri* produced single bands at ~600bp (Figure 3.15), the sequences of which formed two distinct groups. The first group contained specimens from NO (n=9), with the ITS1 sequence 483bp in length, and the second of specimens from LP (n=10), with ITS1 sequences 490bp in length (Figure 3.20). Within each group, the sequences were identical, but there were many nucleotide differences between the two groups. The NO and LP ITS1 sequences are almost identical for the first 300bp and last 130bp, with the variable regions concentrated in between them. The two groups have 451/490 (92.0%) nucleotides in common, with 39 variable sites, or 8.0% sequence divergence, between them.

3.4. Discussion

High levels of intraspecific variation within the mitochondrial and ribosomal markers examined in this study indicate the presence of two cryptic species within the six species (identified based on morphological data) from Canada that were examined using molecular data. These include *An. punctipennis* (BC vs. ON species) and *An. walkeri* (LP vs. NO species), which are based on geographic location, and not based on morphological data (i.e., *An. perplexens* and *An. smaragdinus*) as expected. The results of the three markers examined (COI, ITS2, and ITS1) were in good agreement, but, due to the difficulty in amplifying and sequencing the ITS1 region in all species examined, data sets for COI and ITS2, the most common markers used in taxonomic and phylogenetic investigations of *Anopheles* mosquitoes, were more robust. Levels of intraspecific variation ranged from absent to low in some species (e.g., *An. barberi* and *An. freeborni*) to moderate or high in others (e.g., *An. earlei* and *An. walkeri*, respectively). Sometimes variation was consistent among all markers examined, either all uniform (e.g., *An. freeborni*) or all with significant intraspecific variation (e.g., *An. walkeri*), and others

```
1NI213_3_quad_ITS1        --------------GCGGTCTTCATCGATCCATGAGCCGAGTGATCCCCTGCCTAGGGTT 46
1NI91-7_quad_ITS1         GGAATTTCTTGTAAGCGCTGGTCATTAG--CTAGCGCTGAATACGTCCCTGCCTTTTGTA 58
                                        *** *  ****     *  * ** ** *    ********   **

1NI213_3_quad_ITS1        -ATTCTGTGTGTT--TGTCTCCGTTCGTATTTGTGGCTCCATGTATGTATCTCCCAGAGT 103
1NI91-7_quad_ITS1         CACACCGCCCGTCGCTACTACCGATGGATTATTTAGTGAGGTCTCTGGAGGTGATCGTTC 118
                           *  * *   **   *    *** * *  * * * *     * * ** *  *    *

1NI213_3_quad_ITS1        GCTGATCACCACACCACAGGATATCCCCGTTGGAGCCATCATCCAATGGTGTGTGTTGTG 163
1NI91-7_quad_ITS1         GCAGGCTCCCTCGTGGAGTAGCGTCTGCTTTGCTGAAGTTGACCGAACTTGATGATTTAG 178
                          ** *    ** *           **  * ***  *   *   ** *   **    **  *

1NI213_3_quad_ITS1        TGCGATCAGCACGTACGTGTCGCTTGCCGTCGTTGCACTCCATGCGGAACACATACGACA 223
1NI91-7_quad_ITS1         AGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAAC---CTGCGGAAGGATCATTACT 235
                           *  *  *  *          * ** **** * ** **    *******     *  **

1NI213_3_quad_ITS1        CTTGG-CACGGTCGGACCACGGTGAGCTCCAGCTGGCGGGCCTTCATG-TTCCCGAATTC 281
1NI91-7_quad_ITS1         GATGTTCCAACCCAAACCGAGGTGA-------CCGGAGGGTCATCATCATTCGCGTGTCG 288
                            **  *     *  ***  *****       * ** *** * ****  *** **  *

1NI213_3_quad_ITS1        CCCAGACTTAGAACGTTGAACCGACCGTTACAACCGAACCCCGTTCGATTGCTTCCGACA 341
1NI91-7_quad_ITS1         TCGAAACGCAAACAGGTCGGCGTGCCG-TGTTTGCGACGACCATTAGGTCG-TTTCGCTA 346
                           * * **  * *  * *   *   *** *     ***   ** ** * * * ** **  *

1NI213_3_quad_ITS1        CAGTATGTTAGACGTTTGGGCACCATTTCGTGTCGCCCGGAGCTATGCTATTGTTCCCGA 401
1NI91-7_quad_ITS1         TGGTGGGTTT--CCCTCGGGAACAATAGCATAGCTCCGGGCGACACGAAATGGTGCCCAA 404
                            **  ***   *  * *** ** **  * *  * ** ** *  * *  ** ** *** *

1NI213_3_quad_ITS1        GGG--AAACCCACCATAGCG-AAACGACCTAATGGTCGTCG-CAAACACGGCACGCCGAC 457
1NI91-7_quad_ITS1         ACGTCTAACATACTGTGTCGGAAGCAATCGAACGGGGTTCGGTTGTAACGGTCGGTTCAA 464
                            *   ***  **  *  ** ** * * * ** **   ***      ****   *   *

1NI213_3_quad_ITS1        CTGTTTGCGTTTCGACGACACGCGAATGATGATGACCCTCCGGTCACCTCGGTTTGGGTT 517
1NI91-7_quad_ITS1         CGTTCTAAGTCTGGGGAATTCGGGAAC-ATGAAGGCCCGCCAG----CTGG--------- 510
                          *  * *  ** * *   *  ** ***  **** * *** ** *    ** *

1NI213_3_quad_ITS1        GGAACATCAGTAATGATCCTTCCG--CAGGTTCACCTACGGAAACCTTGTTACGACTTTT 575
1NI91-7_quad_ITS1         AGCTCACCG----TGGTCCGACCGTGCCAAGTGTCGTATGTGTTCC--GCATGGAGTGCA 564
                           *  ** *     ** ***  ***  *    *  * ** *    **  *    ** *

1NI213_3_quad_ITS1        ACTTCCTCTAAATCATCAAGTTCGGTCAACTTCAGCAAAGCAGACGCTACTCCACGAGGG 635
1NI91-7_quad_ITS1         ACGACGGCAAG--CGACACGTACGTGCTGATCGCACACAACACACACCATTGGATGATGG 622
                          **  *  * *   *  ** ** **  *   *    ** * ** ** * * *  * ** **

1NI213_3_quad_ITS1        AGCCTGCGAACGATCACCTCCAGAGACCTCACTAAATAATCCATCGGTAGTAGCGACGGG 695
1NI91-7_quad_ITS1         CTCCAACGGG-GATATCCTGTGGTGTGG-----TGATCAGCACTCTG--GGAGATACATA 674
                            **  **   ***  ***   * *          ** * *  ** *  * **  **

1NI213_3_quad_ITS1        CGGTGTGTACAAAAGGCAGGGACGTATTCAGCGCTAGCTAATGACCAGCGCTTACAAGAA 755
1NI91-7_quad_ITS1         CATGGAG--CCACAAATACGAACGGAGACAAACACACAGAATAACCCTAG---GCAGGGG 729
                          *   * *  * * *   * * *** *  **     *   *** ***   *

1NI213_3_quad_ITS1        A 756
1NI91-7_quad_ITS1         -
```

Figure 3.19. Clustal alignment of *An. quadrimaculatus s.l.* ITS1 sequences. Specimen lNI91-7 represents the first group (n=35, NI, WE, OT), and lNI213-3 the second (n=10, NI and WE).

```
1NO7-4_walkeri_ITS1     GGAATTTCTAGTAAGCGCTGGTCATTAACTAGCGCTGATTAAGTCCCTGCCCCTTGTACA 60
LP01-12_walk_ITS1       GGAATTTCTAGTAAGCGCTGGTCATTAACTAGCGCTGATTAAGTCCCTGCCCCTTGTACA 60
                        ************************************************************

1NO7-4_walkeri_ITS1     CACCGCCCGTCGCTACTACCGATGGATTATTTAGTGAGGTCTCTGGAGGTGATTCATGCG 120
LP01-12_walk_ITS1       CACCGCCCGTCGCTACTACCGATGGATTATTTAGTGAGGTCTCTGGAGGTGATTCATGCG 120
                        ************************************************************

1NO7-4_walkeri_ITS1     CACACCCCTCGCGGGCGCGTCTGCTTCGCTGAAGTTGGCCGAACTTGATGGTTTAGAGGA 180
LP01-12_walk_ITS1       CACACCCCTCGCGGGCGCGTCTGCTTCGCTGAAGTTGGCCGAACTTGATGGTTTAGAGGA 180
                        ************************************************************

1NO7-4_walkeri_ITS1     AGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATGGAGAACT 240
LP01-12_walk_ITS1       AGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATGGAGAACT 240
                        ************************************************************

1NO7-4_walkeri_ITS1     AGATGCATGCTGCATGCATTTACTCGAACGATCGTGTCGACTAGGAGAAGT--------A 292
LP01-12_walk_ITS1       AGATGCATGCTGCATGCATTTACTCGAACGATCGTGTCGACTAGGAGCAGTGCGGTGGTA 300
                        *********************************************** ***        *

1NO7-4_walkeri_ITS1     TATGTGTTGCTAT--GCGTTTGCCATGGTCAACCGAAGTCAAGAGTACACACTGCTCTGG 350
LP01-12_walk_ITS1       TACGTGATGCTGTGCGCGTTTGCCGAAGTCAAGCGTG--CGTACACACTGCTTGCTCTGG 358
                        ** *** **** *  *********   ***** **    *      **    ********

1NO7-4_walkeri_ITS1     TATCGTACCCTGCGGCGTCTAATTGGACCGTCTGATGCATATACAGACAGGTGTGGTCGC 410
LP01-12_walk_ITS1       TAACGTACCCTGCGGCGTCTAATTGGACCGTCTGATGCATATACGGACAGGTGTGGTCGC 418
                        ** ***************************************** ***************

1NO7-4_walkeri_ITS1     TTACTGGACGTGGCAGGTGGTGCGCCCACTTGGAAGACGGATATATACACGGAACAACTT 470
LP01-12_walk_ITS1       TTACTGGACGTGGCAGGTGGTGCGCCCACTTGGAGGATG-ATGTATACACGGAACAACTT 477
                        ********************************** ** * ** *****************

1NO7-4_walkeri_ITS1     CCCTAGGCAGGGG 483
LP01-12_walk_ITS1       CCCTAGGCAGGGG 490
                        *************
```

Figure 3.20. Clustal alignment of LP and NO *An. walkeri* ITS1 sequences. Specimen lNO7-4 represents the first group, containing specimens from NO (n=9), and LP01-12 the second group, containing specimens from LP (n=10).

revealed variation in one or more, but not all, of them (e.g., *An. earlei*).

Intraspecific variation was low in the COI and ITS2 sequences of *An. barberi.* Average COI sequence divergence levels were 0.1% in *An. barberi,* with a maximum divergence of 0.4% among the 16 specimens examined (Table 3.5). Variation in the ITS2 sequences of *An. barberi* was very low, with only 1 nucleotide difference in one specimen examined (n=18) (Table 3.9). These results are as expected since all specimens were from the same woodlot in Niagara and likely represent a single breeding population. The inability of the ITS1 primers designed for this study to amplify the ITS1 region in this species is probably due to its relatively distant phylogenetic relationship to the species upon whose 18S and 5.8S sequences the primers were designed. *An. barberi* is unique morphologically and in larval habitat, the only species examined that belongs to the Plumbeus Group, with all others found within the Maculipennis or Punctipennis Groups (Harbach 2004) (Figure 1.6). While the 18S, 5.8S, and 28S sequences are more highly conserved than the ITS sequences located between them, sufficient nucleotide differences likely exist in one of the primer binding sites such that it goes unrecognized by the ITS1F or ITS1R primer. *An. barberi* was the most derived species of those examined in neighbour-joining analyses of both COI and ITS2 (Figure 3.9 and 3.14).

Although *An. earlei* has the broadest distribution in Canada of all species examined, intraspecific variation in COI and ITS2 sequences was relatively low. Although specimens from a wide range of locations (BC, NO, OT, RQ, and NF) were included in analysis of COI sequences (n=40), maximum sequence divergence level within *An. earlei* was only 0.6% (Table 3.5). There was weak support for a cluster of specimens that consisted of all 4 BC *An. earlei* individuals, but it included 3 of the 8 AL specimens as well (Figure 3.4). There were other similar small clusters of individuals with shared nucleotide changes, but they were not geographically significant. The large size of ITS2 in *An. earlei* has also been observed in the related species *An. beklemishevi,* a Russian member of the *An. maculipennis* complex (Kampen 2005). Kampen (2005) found that alignment of the 638bp *An. beklemishevi* ITS2 sequence with those of other members of the complex was only possible in the first ~335bp and the last ~150bp, similar to that of the *An. earlei* ITS2 results of this study. This suggests that *An. earlei*

may be more closely related to Palearctic members of the *An. maculipennis* complex than Nearctic ones.

Although sequencing of the entire ~800bp ITS2 region in *An. earlei* was not possible, analysis of the edited 318bp sequences revealed two distinct groups; the first contained all BC specimens and the second contained specimens from all remaining regions (RQ, NO, OT, and NF) (Figure 3.11). ITS2 sequences were identical within each group, and differed by 2 nucleotides between them (Table 3.9), suggesting perhaps some degree of isolation between *An. earlei* specimens on either side of the Rocky Mountains (as in *An. punctipennis*, see below). However, sufficient evidence for potential cryptic species within *An. earlei* (i.e., western versus eastern forms) based on COI and ITS2 was not present.

Analysis of ITS1 in *An. earlei* was even more difficult than ITS2 due to both the extremely long length of the sequence (~4000bp) and the inability of the sequencing reaction to successfully sequence the first ~750bp in each direction. The difficulty in sequencing may be due to intraindividual variation in ITS1 (i.e., different ITS1 sequences present within the rDNA array). Analysis of ITS1 in other *Anopheles* species has revealed intragenomic variation, due primarily to the presence of internal repeats, which vary in size and number within each ITS1 subunit in the array, resulting in different ITS1 lengths and sequences within the same individual (Bower *et al.* 2008; Bower *et al.* 2009; Paskewitz *et a.* 1993). Therefore, detailed analysis through cloning of the ITS1 region in *An. earlei* could potentially reveal population level information to help to understand the phylogeographic history of this species in Canada and North America. However, the presence of multiple ITS1 bands in some species could represent pseudogenes located outside the rDNA genes. Similar phylogenetic relationships between *An. earlei* and the remaining species were observed in neighbour-joining analyses of COI and ITS2 (Figure 3.9 and 3.14). COI data revealed strong support for *An. earlei* and *An. freeborni* being sister taxa, but this relationship was less clear based on ITS2 data. This is likely due to editing of the 800bp *An. earlei* ITS2 sequences to a shorter 318bp portion for analysis.

Similar to *An. barberi*, intraspecific variation in the COI and ITS2 sequences of *An. freeborni* was very low. Average COI sequence divergence was 0.0%, with a maximum of 0.2%, among the *An. freeborni* individuals examined (n=30) (Table 3.5).

The ITS2 regions of all *An. freeborni* specimens examined (n=26) were identical throughout the entire 422bp sequence (Table 3.9). While the ITS1 region of *An. freeborni* is similar to that of *An. earlei* (single bands, ~4500bp and ~4000bp, respectively), sequencing ITS1 in *An. freeborni* was successful. Combining the ~750bp ITS1F and ITS1R sequences, a total of only 10/1557 nucleotide differences were observed (n= 9), resulting in an intraspecific sequence divergence level of 0.6% in these sections of the total ~4500bp ITS1 sequence. Despite sampling from geographic locations throughout most of southern British Columbia, most of the *An. freeborni* specimens examined were from the same site in Kamloops, but since it is known from a limited distribution in BC (Figure 1.10), *An. freeborni* is likely a single species throughout its range in Canada.

Another species with a relatively restricted distribution in Canada is *An. quadrimaculatus s.l.*, the northern limit of which is extended into southern Ontario and Quebec. The average COI sequence divergence level among *An. quadrimaculatus s.l.* specimens (n=60), collected from various regions in Ontario (NI, WE, and OT), was 0.6%, with a maximum of 1.2% (Table 3.5). There was a high number of unique haplotypes and variable sites among the 806bp sequence (46 and 52, respectively) (Table 3.9). Distinct clusters based on either collection location or morphological species identification (*An. quadrimaculatus s.s.* or *An. smaragdinus*) were not observed in NJ analysis of COI sequences (Figure 3.7), and all of the 73 *An. quadrimaculatus s.l.* ITS2 sequences obtained in the study were identical (Table 3.9). Since differences are present in the ITS2 sequences of members of the *An. quadrimaculatus s.l.* species complex (Cornel *et al.* 1996), these results suggests that the morphological characters used to discriminate these species (as described in Darsie and Ward 2005) are not applicable here near the northern limit of their ranges. Both COI and ITS2 data indicate that only *An. quadrimaculatus s.s.* occurs in Canada, as predicted by Levine *et al.* (2004) in their model for potential distribution of the members of the *An. quadrimaculatus* complex.

The ITS1 sequences of *An. quadrimaculatus s.l.*, however, did reveal intraspecific variation, the source of which is not clear. *Anopheles quadrimaculatus s.l.* ITS1 sequences formed two distinct groups, which again did not correspond to either collection location or initial morphological identification as either *An. quadrimaculatus* or *An.*

smaragdinus. Within each group, the sequences are identical, but the first group (NI, WE, and OT specimens; n=35) differs from the second group (NI and WE specimens; n=10) by 26 nucleotides in size and a sequence divergence of ~56% (Figure 3.19). This is the highest level of intraspecific variation in any marker or species measured in the study, and does not agree with the results of both COI and ITS2 datasets. Therefore, further studies are required to determine the source of ITS1 variation in this species.

Anopheles punctipennis, as identified based on morphology, has a sporadic distribution in Canada (Figure 1.11), and exhibited some of the highest levels of intraspecific variation observed among all species examined. Both COI and ITS2 data revealed the same cryptic intraspecific clusters within *An. punctipennis* (i.e., BC versus ON specimens) (Figures 3.9 and 3.14). Using the 5′ 650bp barcoding region of COI, Cywinska *et al.* (2006) found that sequence divergences for conspecific individuals (all from Ontario) was usually less than 0.5%, much lower than the 2.7% observed between the BC and ON groups of *An. punctipennis* (Figure 3.6). ITS2 sequences are known to be species-specific in *Anopheles* (Collins and Paskewitz 1996), even among recently derived species (such as the members of the *An. quadrimaculatus* species complex which range in length from 289 to 329bp and are easily distinguished based on nucleotide sequence). Therefore, the high level of ITS2 sequence divergence between the BC and ON *An. punctipennis* groups (7.7%) (Figure 3.12) provides further evidence for the presence of a cryptic species within this taxon. In a phylogeny of Nearctic *Anopheles* species based on the D2 variable region of the 28S rDNA, Porter and Collins (1996) examined *An. punctipennis* specimens from California and Wisconsin and Illinois and describe them as sister taxa, which they called *An. punctipennis* W and *An. punctipennis* E. Therefore, the COI and ITS2 results of this study and the D2 results of the Porter and Collins (1996) are in good agreement and provide further evidence for the presence of a cryptic species within this taxon.

ITS1 results, however, were complicated by the presence of multiple ITS1 PCR products from most specimens examined that were identified morphologically as either *An. punctipennis* or *An. perplexens*. Intraindividual variation in the ITS1 region has been observed in other anopheline species, and sequencing of clones containing single ITS1 subunits revealed geographic patterns in the number and type and internal variant repeats

within them that were used to examine the population structure of this species throughout its range (Bower *et al.* 2008). Therefore, analysis of the ITS1 region through the sequencing of individual clones might also be useful in population-level studies of *An. punctipennis*. Although ITS1 sequence data was not obtained for this species through the current study, the only specimens that resulted in single ITS1 bands were those from BC, providing further evidence for the presence of cryptic species in this taxon. However, the presence of suspected cryptic species, *An. perplexens*, could not be confirmed based on COI or ITS2 data. The similar result of low divergence level between specimens from Ontario identified as *An. punctipennis* or *An. perplexens* (using morphological data) was obtained during previous studies involving the barcoding region of COI (A. Cywinska, *pers. comm.*). The possibility that *An. perplexens* and *An. punctipennis* are, in fact, truly reproductively isolated species, but they don't differ in any of these molecular markers, is very low, and proof that the morphological characters associated with each species, particularly the size of the pale-coloured wing spot in adults, are highly variable in certain regions of Ontario. In NJ analyses of COI and ITS2 sequences (Figures 3.9 and 3.14), *An. punctipennis* (including those identified as *An. perplexens*) formed two groups (BC and ON), which were most closely related to *An. quadrimaculatus s.l.* and the Nearctic members of the *An. maculipennis* complex (i.e., *An. earlei* and *An. freeborni*).

Finally, *An. walkeri* had the highest levels of intraspecific variation observed within COI and ITS2 of all species examined. Because a limited number of specimens from only two main collecting regions were available for analysis, the presence of cryptic species within *An. walkeri* was not expected. However, significant sequence divergence levels were observed in all three markers analysed, with specimens forming distinct clusters based on collection location (i.e., NO and LP) (Figures 3.8, 3.13 and 3.20). Sequence divergence in the COI sequences between the two groups was ~7%, considerably higher than would be expected if all specimens represented a single species (Hebert *et al.* 2003; Cywinska *et al.* 2006). The ITS2 sequences of *An. walkeri* specimens (identified based on morphological characters) required editing due to difficulty sequencing in the reverse direction, but sequence divergence within the 231bp section examined was ~32% between the two groups. The ITS1 region was easily amplified and sequenced within morphologically identified *An. walkeri*, sequence

divergence between the NO and LP groups was ~8%. These data strongly support the presence of a cryptic species within this taxon.

The distribution of *An. walkeri* extends north into Canada from Saskatchewan to Quebec and the Maritimes, but it presence on Manitoulin Island (NO) and north in central Ontario has not yet been recorded (Figure 1.13). The distribution of *An. punctipennis* is similar, with a northern range limit near Manitoulin Island (Figure 1.11), and specimens identified morphologically as *An. punctipennis* from NO were included in analyses of both COI and ITS2 sequences. However, similar clusters of individuals were not present with specimens identified morphologically as *An. punctipennis* (i.e., NO vs. other regions in ON), suggesting that the island nature of Manitoulin alone may not be the source of variation within *An. walkeri*. Further studies are required to determine the range and further characterize *An. walkeri* and its newly recognized sibling species. In neighbour-joining analyses of both COI and ITS2 sequences (Figures 3.9 and 3.14), other than *An. barberi*, *An. walkeri* was the most distantly related species to the remaining marsh anophelines.

This is the first study to examine the 800bp section of the COI located at the 3′ end of the gene in *Anopheles* mosquitoes from Canada, the results of which agree with those of previous molecular studies. Cywinska *et al.* (2006) examined the 650bp barcoding region located at the 5′ end of the COI gene in mosquito species from Ontario, including four anophelines. In both studies, all specimens examined formed tight species clusters and hypotheses of phylogenetic relationships among species based neighbour-joining analysis of the Kimura-2-parameter (K2P) distances were similar. The 650bp barcoding COI sequence showed *An. earlei* to be most closely related to *An. punctipennis,* followed by *An. quadrimaculatus* then *An. walkeri* (Cywinska *et al.* 2006). However, Cywinska *et al.* (2006) did not include *An. freeborni* and *An. barberi*, or specimens from British Columbia or Manitoulin Island, and thus did not observe the potential cryptic species in *An. punctipennis* or *An. walkeri* that were revealed in the current study. Based on the 800bp COI sequence, *An. earlei*, *An. freeborni* and *An. punctipennis* are most closely related, with *An. punctipennis* E (ON) and *An. punctipennis*

W (BC) forming distinct groups, followed by *An. quadrimaculatus s.l.*, and more distantly, *An. walkeri* (NO and LP) and *An. barberi.*

These results are in agreement with those of another study of Nearctic anophelines based on the D2 variable region of 28S rDNA (Porter and Collins 1996). In this study, *An. earlei*, *An. freeborni*, *An. punctipennis* E and *An. punctipennis* W form one group, and *An. quadrimaculatus* species A, B, C, and D form another, both groups being distantly related to *An. walkeri*, the most divergent of all species examined (although *An. barberi* was not included) (Porter and Collins 1996).

In general, the proposed phylogenetic relationships among the anopheline species that occur in Canada based on COI and ITS2 data are similar, but a few differences are apparent (Figures 3.9 and 3.14). *An. walkeri* and *An. barberi* are the most divergent species in both trees, but the close relationship between *An. earlei*, *An. freeborni*, and *An. punctipennis* (E and W) in the COI tree is not as clear in the ITS2 tree. This is likely due in part to the requirement for editing of the 800bp ITS2 sequence in *An. earlei.* Most of the ITS2 region is not translated or involved in ribosomal processing and, thus, not subject to functional constraints so insertions and deletions are free to accumulate. Therefore, ITS2 may be less suitable for hypotheses of phylogenetic relationships of more distantly related species.

3.5. Summary and Conclusions

While analysis of mitochondrial and ribosomal DNA variation among the *Anopheles* species of Canada revealed the presence of cryptic species, they were based on geographic location, and not on morphological identifications (i.e., *An. perplexens* and *An. smaragdinus*) as expected. In particular, while both COI and ITS2 provided evidence for a cryptic species within *An. punctipennis,* the two groups differed based on collection location (i.e., BC vs. ON), and not morphological variation in the size of the pale-scaled wings spots (adults) or the state of setae 2-IV and 2-V (larvae) (i.e., *An. punctipennis* vs. *An. perplexens*). ITS1 data seem to confirm these results, with all BC *An. punctipennis* producing single ITS1 bands and all ON *An. punctipennis* producing multiple bands (2-5 per individual). Similar results based on the D2 variable region of 28S data (Porter and

Collins 1996) suggest that, in North America, *An. punctipennis* may be a complex of two or more species, including an eastern and western species.

The results of COI, ITS2, and ITS1 data also indicate the presence of a cryptic species within *An. walkeri*. All three markers were significantly different between the Manitoulin Island and Long Point Provincial Park specimens. Preliminary morphological examination of these individuals did not reveal any obvious morphological differences between the two groups, and the Manitoulin Island specimens represent first records for that location. Further studies are required to characterize the differences between *An. walkeri* and its newly recognized sibling species.

With respect to the suspected cryptic species *An. smaragdinus,* COI, ITS2, and ITS1 could not confirm its presence in Canada. Despite the large number of specimens that key out to this member of the *An. quadrimaculatus* complex, specimens identified morphologically as *An. smaragdinus* did not differ from those identified as *An. quadrimaculatus s.s* based on ITS2 sequences. This suggests that the morphological characters used to distinguish these species in the USA may not be applicable here at the northern limit of their ranges.

COI and ITS2 data also provided evidence that both *An. quadrimaculatus s.s.* and *An. freeborni* likely occur as single species throughout their range in Canada. Because all *An. barberi* specimens were obtained from the same woodlot in Niagara, the level of intraspecific variation observed in this species may not be a reflection of the actual variation throughout its range in southern Ontario and Quebec. Finally, although *An. earlei* has the broadest distribution in Canada, and samples were obtained from a wide variety of locations, COI and ITS2 did not reveal the presence of cryptic species within this taxon, although there were minor differences between specimens from BC and those from the remaining collection sites. This suggests that the Rocky Mountains may cause some degree of isolation between eastern and western populations of *An. earlei* on either side, but not as much as that observed between populations of *An. punctipennis*. Therefore, the analysis of molecular data proved a valuable perspective from which to examine the cryptic species status of *Anopheles* mosquitoes from Canada.

Chapter Four:
Ecological Analyses of the Larval Habitats of *Anopheles* in Canada

4.1. Introduction

Ecological traits have long been known as potential distinguishing characters among closely related species of *Anopheles* mosquitoes. In his paper on race formation and speciation in mosquitoes, Kitzmiller (1959) describes the "Anophelism without malaria" problem and subsequent discovery that the primary vector, *An. maculipennis*, was in fact a complex of morphologically similar species that varied widely from one another in ecological associations, such as larval habitat (e.g., salinity tolerance) and host association. Crossing experiments and polytene chromosome data confirmed the specific status of suspected cryptic species by showing that reproductive isolation existed in varying degrees among various populations of *An. maculipennis* throughout Europe (Kitzmiller 1959). A similar study involving hybridization experiments in *An. gambiae* also revealed ecological differences in salinity tolerance (e.g., fresh versus salt water larval habitats) among "populations" from various regions in Africa, which indicated the probable existence of distinct biological species within this taxon (Paterson 1962). Both *An. maculipennis* and *An. gambiae* are now known as complexes of isomorphic species, distinguishable based on multiple types of data, including polytene chromosome (Frizzi 1947; Coluzzi *et al.* 1977) and molecular (ITS2) data (Proft *et al.* 1999; Paskewitz *et al.* 1993), but it was ecological data that led to the discovery of cryptic members within each of these species complexes.

Ecological data is an important aspect of insect systematics, and modern molecular methods of species identification have provided a theoretical foundation for detailed studies of ecological characters to aid in accurate species delineation. For example, the neotropical butterfly *Astraptes fulgerator* (Lepidoptera: Hesperiidae) was once believed to be a single species that was common, variable and had a very broad distribution (from the southern USA to northern Argentina), found in near desert to near deep rainforest habitats, at a variety of elevations, and in urban or pristine environments (Hebert *et al.* 2004). However, analysis of the barcoding region of the *cytochrome oxidase* I gene (COI) revealed the presence of at least ten cryptic species that, once

recognized, led to discovery that these largely sympatric species had different caterpillar plant foods, larval morphology, and ecosystem preferences accompanied by only very slight differences in adult morphology, including genitalia (Hebert *et al.* 2004).

In a similar study of 16 species of tropical parasitoid flies (Diptera: Tachinidae) that were believed to be generalists based on the high number of host species (caterpillars) from which they had been reared, DNA barcoding led to the discovery of numerous cryptic species within the 16 supposedly generalist species, many of which were, in fact, specialist species (i.e., reared from only one species of caterpillar) (Smith *et al.* 2007). Four different ecological patterns were revealed among the 16 species: 1) a single generalist species, 2) a pair of morphologically cryptic generalist species, 3) a complex of specialist species plus a generalist, and 4) a complex of specialists with no remaining generalist (Smith *et al.* 2007). This example illustrates the importance of ecological traits in the description of cryptic species.

Within *Anopheles* mosquitoes, ecological characters commonly used to distinguish among members of species complexes include host association (e.g., human versus other mammal or bird biters) and larval habitat associations (e.g., fresh water versus salt water, vegetation type) among others. In malaria endemic regions, many potential vector species belong to isomorphic species complexes whose members differ in their ability to transmit the malarial parasite, which is often a result of differences in ecological traits. Isomorphic members of the *An. gambiae* complex have been the focus of intense research using multiple types of data to find a simple, reliable method for identifying each species that would allow vector control efforts to target the correct species. Once species identification using polytene chromosome data became possible, the ecological differences among species of the *An. gambiae* complex in Africa could then be determined. Larvae of two species, *An. merus* and *An. melas*, occur in salt-water habitats of the eastern and western coastal regions of Africa, respectively, whereas the larvae of four other species occur in widely distributed fresh-water habitats of non-coastal regions (Coluzzi *et al.* 1977). *Anopheles gambiae* and *An. arabiensis* are the most anthropophilic members of the complex with the widest distributions, and therefore represent the primary vectors of malarial parasites in the regions where they occur (Coluzzi *et al.* 1977). However, they have different distribution limits and seasonal

prevalence, such that *An. gambiae* predominate in forest and humid savanna regions and *An. arabiensis* in arid savannas and steppes (Coluzzi *et al.* 1977). In addition, in areas where they occur sympatrically, *An. arabiensis* increases in relative frequency during the dry season (Coluzzi *et al.* 1977). Because a fifth species, *An. quadriannulatus,* exhibits marked zoophily, it is not implicated in the spread of human malaria and therefore is not considered to be of medical importance (Coluzzi *et al.* 1977). The larvae of a sixth species, *An. bwambae,* are specific to the pools of mineral springs found only in the Semliki forest in Uganda (Coluzzi *et al.* 1977; Coluzzi *et al.* 2002). This example illustrates how larval habitat correlates can be an important character set when investigating the status of cryptic species.

Most of the known information regarding the ecological traits of *Anopheles* species present in Canada can be found in Mosquitoes of North America, north of Mexico (Carpenter and LaCasse, 1955), and, from a Canadian perspective, in The Mosquitoes of Canada (Wood *et al.* 1979). While these authors do include general bionomical data based on personal observations for certain life stages and/or species, the type of data described (e.g. larval habitat, seasonal prevalence, etc.) is inconsistent among species and sometimes based on studies conducted only in the USA. The known larval habitats for all seven previously recognized anopheline species (based on morphological data) present in Canada (see Chapter One) are listed in Table 4.1 (Carpenter and LaCasse 1955; Wood *et al.* 1979; Thielman and Hunter 2007). Four species are sometimes referred to as the "marsh-breeding" species including *An. earlei, An. punctipennis, An. quadrimaculatus s.l.*, and *An. walkeri*, but larvae of *An. freeborni* in British Columbia are also sometimes found in marshes (Wood *et al.* 1979).

In addition to these seven species, identified based on morphological characters, two cryptic species were suspected to occur in Canada: *An. perplexens* (currently known only from the USA and morphologically similar to *An. punctipennis*) and *An. smaragdinus* (a cryptic member of the *An. quadrimaculatus* complex from the USA). These species were identified in 2005-2009 field collections based on morphological identifications of adults and larvae following the key in The Identification and Geographical Distribution of Mosquitoes of North America, north of Mexico (Darsie and Ward 2005). *Anopheles perplexens* is closely related to *An. punctipennis,* from

Table 4.1. Larval habitat descriptions for the seven *Anopheles* species previously known from Canada based on morphological data and the three cryptic species (indicated by *) whose presence in Canada was suspected based on preliminary morphological identifications (see Chapter Two) and the close proximity of their ranges to the Canadian border (see Chapter One) (Carpenter and LaCasse 1955; Bellamy 1956; Wood *et al.* 1979; Reinert *et al.* 1997).

Species	**Larval Habitat Associations**
An. barberi	found primarily tree holes, rarely in artificial containers
An. crucians s.l.	ponds, lakes, swamps, and semi-permanent and permanent pools, in acidic water, usually with floating or emergent vegetation
An. freeborni	pools and sloughs formed by creeks and large marshes, usually with emergent vegetation
An. earlei	cold clear water in shallow margins of semi-permanent and permanent ponds overgrown with floating and emergent vegetation, woodland pools, associated with other anophelines
*An. occidentalis**	permanent pools, ponded creeks, shallow margins of ponds and hillside seepages along the Pacific West coast
*An. perplexens**	clear calcareous springs and streams that issue from them, lime sink holes with shaded clear water covered with duckweed
An. punctipennis	largest variety of habitats, pools and ponds with floating, emergent or no vegetation, rock pools, tree holes, artificial containers, puddles, usually associated with other anophelines
An. quadrimaculatus s.s.	various habitats, marshy edges of rivers lakes, sluggish streams, canals, usually in permanent fresh water, associated with other marsh-breeding species
*An. smaragdinus**	permanent swamps with filtered sunlight and limited aquatic vegetation
An. walkeri	ponds with cattails and emergent vegetation, associated with other marsh-breeding species

which it can be distinguished chromosomally, but not reliably morphologically (except in the egg stage) (Bellamy 1956; Linley and Kaiser 1994). *Anopheles smaragdinus* is a relatively recently recognized species in the *An. quadrimaculatus* complex, identifiable by numerous methods including polytene chromosomes, molecular (ITS2) data, and minor differences in egg, larval, and adult morphology (Reinert *et al.* 1997). While *An. quadrimaculatus s.s.* appears to be opportunistic in habitat selection and larvae are known from a wide variety of habitats, *An. smaragdinus* larvae are reported to be associated with permanent swamps with filtered sunlight and limited aquatic vegetation (Reinert *et al.* 1997). Similarly, *An. punctipennis* is known from a wide variety of larval habitats throughout North America, but the larvae of *An. perplexens* are specific to alvar habitats (i.e., exposed limestone), such as the circular, limestone sinkholes present at the type locality in Georgia, USA (Bellamy 1956).

To investigate larval habitat and species associations of *Anopheles* species in Canada, bionomic data from larval dipping collections were recorded and larval and adult specimens reared from those collections were identified to species. Thus, the analysis of ecological data represents a third approach to investigating the cryptic species status of *Anopheles* species from Canada. This study represents the first detailed treatment of larval habitat data associated with anopheline species here near the northern limits of their ranges. The objectives of this ecological component of the overall study are to look for ecological evidence of cryptic species, and to provide updated Canadian bionomic information, including larval habitat and species associations, for each *Anopheles* species known from Canada.

4.2. Materials and Methods

Larvae and pupae were collected from a wide variety of habitats and locations in Canada (Figures 2.11, 2.12 and 2.13). Specimen collection methods, preservation, and identification were described in Chapter Two. All specimens were identified to species based on morphological data using the key of Darsie and Ward (2005), and although results of molecular analyses suggested that specimens identified morphologically as *An. perplexens* and *An. smaragdinus* were simply morphological variants of *An. punctipennis*

and *An. quadrimaculatus s.s.* respectively, original species names were kept for analyses of ecological data to determine whether ecological factors were related to the morphological differences could be observed.

A data collection form was designed and used to record the larval habitat data associated with each mosquito-positive collection (types listed in Table 1 of Appendix II). The ecological data for all larval dipping collections are maintained in an online database designed specifically for this research, which will be made available to the public upon completion of all degree requirements (see Appendix II). Each larval dipping collection consisted of a minimum of 20 dips (no maximum) to obtain at least 100 specimens per site, if possible. For each larval dipping collection, larval habitat was classified as one of ten possible water body types (called "container type" in the database) (Table 4.2), and the type of emergent and floating vegetation, if present, was recorded (Table 4.3).

Table 4.2. Larval habitat water body type classifications used for all *Anopheles*-positive larval dipping collections. Definitions as used in the current study are provided.

Water Body Classification	**Definition**
alvar/quarry	water surrounded by limestone walls; old limestone quarries; vegetation usually absent
artificial container	plastic, glass, or metal containers, such as tires, buckets, birdbaths, etc.
bog	dense layer of peat, acidic, water table at or near surface, usually with mosses, shrubs and sedges, trees may be present
ditch/creek	shallow, still or slow-flowing waterways, streams, smaller than rivers, natural or artificial (beside roads)
forest pool	temporary or permanent small pools of water in the forest, includes snowmelt pools, emergent vegetation usually absent
lake/river edge	shallow, slow-flowing waters at the edges of lakes and rivers, usually protected by floating and/or emergent vegetation
marsh	large bodies of water, ~ 1m deep, lots of emergent vegetation such as cattails (*Typha* spp.) present, trees absent
pond	still body of water, smaller than a lake, usually with floating and/or emergent vegetation such as algae
rock pool	small pools of water found on the rocks along the shores of rivers, oceans, and other large, moving, bodies of water
shallow waters	basins, pools, and ponds that are found beside rivers, coastlines, and shoreline, submerged vegetation and floating leaved plants present
slough	large stagnant pools of water, especially as part of a bay, inlet, or backwater, usually with trees and nearly closed canopy
swamp	stagnant or slow-flowing pool, usually covered with trees or shrubbery
tree hole	permanent (deep rot holes) or temporary (shallow rot holes) pools of water in trees

Table 4.3. Vegetation types recorded during 2005-2009 larval dipping collections.

Emergent	**Floating**
cattails (*Typha spp.*)	algae
grasses (Poaceae)	duckweed (Araceae)
common reeds (*Phragmites spp.*)	lily pads (Nymphaeaceae)
pickerel weed (*Pontederia spp.*)	decaying vegetation
arrowhead (Alismataceae)	wood
sedge (*Carex spp.*)	pondweed (e.g. *Elodea spp.*)
terrestrial plants	grasses (Poaceae)
moss/lichen	
shrubs	
blue iris (*Iris versicolor*)	

4.3. Results and Discussion

4.3.1. Overall Species Abundance

A total of 4805 *Anopheles* specimens (2140 larvae and 2665 reared adults) obtained from all larval dipping collections during the 2005-2009 field seasons were reared, preserved, and identified. The most commonly collected species were *An. punctipennis* and *An. quadrimaculatus*, comprising 77.34% of all specimens collected; the least common were *An. walkeri, An. freeborni,* and *An. barberi* (1.37%, 2.37%, and 2.96% percent of all specimens collected, respectively) (Table 4.4). The wide range in abundance among species is likely due in part to actual differences in abundance levels within the larval habitats from which they were collected, and in part to sampling bias, e.g., some regions/water body types sampled more intensely than others, some species a have limited distribution range (e.g., *An. freeborni* occurs only in BC), etc.

The low number of *An. barberi* collected (Table 4.4) was likely related to both the rarity of natural larval habitat in the environment (i.e., deep, elevated tree holes) and the difficulty experienced finding such habitats. Prior to the current study, collection records for *An. barberi* in Ontario included Point Pelee National Park (Smith and Trimble 1973), Guelph (Shipp *et. al.* 1978), and the Perth region (Wood *et al.* 1979). During the 2005 and 2006 field seasons, one larva was collected from a tree hole located in Point Pelee National Park despite considerable effort to find water-filled tree holes in WE (i.e., Point Pelee National Park and surrounding area) and OT (Perth and surrounding area). The other 141 larval and adult specimens were reared from larvae collected from used tires that had been set up in a woodlot in NI by a colleague for an unrelated study of tree hole mosquitoes. Although *An. barberi* larvae have been recorded from artificial containers (Debboun and Hall 1992), their use as a collection tool for this elusive species has not been addressed. The placement of used tires in a woodlot was far more successful in obtaining *An. barberi* specimens throughout the summer than locating *An. barberi*-positive tree holes in a forest.

Table 4.4. Number of *Anopheles* larvae and adults, identified based on morphological characters using the key of Darsie and Ward (2005), obtained by larval dipping collections conducted during 2005-2009 field seasons.

Species	Larvae	Adults	Total	% Total
An. barberi	96	46	142	2.96
An. earlei	250	312	562	11.70
An. freeborni	62	52	114	2.37
An. perplexens	31	135	166	3.45
An. punctipennis	750	1344	2094	43.58
An. quadrimaculatus s.l.	914	708	1622	33.76
An. smaragdinus	1	31	32	0.67
An. walkeri	36	37	73	1.52
Total	2140	2665	4805	

Anopheles earlei was not the most abundant species collected (Table 4.4), but it was collected from the widest variety of geographical locations including British Columbia, Ontario, northern Quebec, and Newfoundland. The collection of *An. earlei* from NF is a first record of this species from the island of Newfoundland. Although considered a marsh species, *An. earlei* is also considered to be more common in wooded areas, a habitat not often sampled during field collections, than in open areas (Wood *et al.* 1979). *Anopheles earlei* occurs much farther north than other anophelines, found throughout the boreal forest (Figure 1.9). It has been called "Canada's National Mosquito" due its association with beavers, as a feeding association study found ~82% of *An. earlei* specimens had fed on beavers (Wright and DeFoliart 1970) and they have been found in large numbers inside a beaver lodge (Hudson 1978). This association with beaver lodges might explain the ability of *An. earlei* to occur farther north than other anopheline species, providing females with ideal (warm, moist) hibernacula in which to overwinter.

The low number of *An. freeborni* specimens collected (Table 4.4) was likely due to both its restricted range in Canada (present only in the Okanagan and Kootenays regions of BC) and the lack of annual sampling in this region. A total of 20 *An. freeborni* larvae and adults were obtained from 23 larval dipping collections conducted during a nine-day collecting trip (21 Jun – 29 Jun) in 2005, whereas the remaining 94 specimens were obtained from 3 larval dipping collections (by a local environmental consulting company) conducted at the same *An. freeborni*-positive pond in Kamloops, BC. Therefore, the low number of *An. freeborni* specimens collected likely does not accurately reflect its actual abundance in the regions where it occurs.

Anopheles punctipennis was the most commonly collected species (Table 4.4). As most larval dipping collections were conducted in southern ON (WE, NI, OT, and NO), and all four marsh anophelines are known from this region, this is likely a good indication of its actual abundance in this region. The relative abundance of specimens identified morphologically as *An. perplexens* was considerably lower, comprising ~3.5% of all specimens collected (Table 4.4). Since *An. perplexens* is known from alvar-type habitats throughout the eastern United States (Figure 1.15), and many similar habitats were located in the NI region during course of this study, we chose to focus collections in

this area during the 2007-2009 field seasons. Although it unlikely that *An. perplexens* and *An. punctipennis* don't differ in the molecular markers examined (see Chapter Three), an attempt to obtain eggs from field-collected *An. perplexens* females was made for confirmation its presence or absence in Canada (see Chapter Two). However, specimens identified as *An. perplexens* were collected in other regions as well. Therefore, the original species identifications were used in the following larval habitat analyses to determine whether the morphological differences observed were related to ecological associations.

Anopheles quadrimaculatus s.l. was the second most commonly collected species (Table 4.4) and, similar to *An. punctipennis*, this is likely a more accurate reflection of the actual abundance of *An. quadrimaculatus s.l.* in the regions where it occurs, for the same reasons mentioned above. The relative abundance of *An. smaragdinus*, the other cryptic species thought to be present in southern ON based on morphological observations, was much lower (0.7% of all specimens collected) because only a subsample of *An. quadrimaculatus s.l.* specimens was identified to the sibling species level. Molecular evidence suggests that individuals identified morphologically as *An. smaragdinus* are simply morphological variants of *An. quadrimaculatus s.s.* (see Chapter Three). All 32 specimens identified as *An. smaragdinus* were collected in NI, but known associated habitats of *An. smaragdinus* (i.e., swamps with filtered sunlight and little vegetation) were not located/sampled in this region, suggesting again that specimens identified as *An. smaragdinus* are likely *An. quadrimaculatus s.s.* variants.

Finally, *An. walkeri* was the least commonly collected of the four marsh species, (Table 4.4), however, the reason for the low number of specimens obtained by larval dipping may not be as straightforward. According to Wood *et al.* (1979), *An. walkeri* is usually the most common anopheline in southern Ontario during the summer months. However, despite being the most intensely sampled area, *An. walkeri* was among the least commonly collected anophelines in southern Ontario during the 2005-09 field seasons (Table 4.4). Possible reasons for this could be that larvae of *An. walkeri* are more easily disturbed during larval collections and/or are able to remain below the surface of the water longer than the other species, or they may have actually decreased in abundance due to a decrease in available larval habitat in this region since the publication of Wood

et al. (1979). Many marsh habitats indicated on topographic maps published in the late 1970s, when located for sampling, had been drained for residential or agricultural use (A. Thielman, *pers. obs.*). Interestingly, there are no previous records of *An. walkeri* from NO, where a cryptic species closely related to *An. walkeri* was discovered based on molecular data (see Chapter Three). Collection of specimens from the type locality near Lake Simcoe, Ontario, and analysis of COI, ITS2, and ITS1 sequences, could determine which population represents the type species and the other the newly recognized species.

4.3.2. Regional Species Abundance

A total of 4805 anopheline specimens were collected from 224 *Anopheles*-positive larval dipping collections conducted in all sampling regions during the 2005-2009 field seasons (Table 4.4). The number of specimens collected varied greatly depending on the species and region of collection. From ~400-2000 specimens were reared from larval dipping collections made in each of five main collecting regions in Canada, i.e., BC, NI, WE, OT, and WE (Figure 4.1); considerably fewer (75 or less) were collected from each of the other six regions, i.e. NF, RQ, AL, HL, FN, and BP. *Anopheles punctipennis* was collected from the greatest number of regions sampled in Canada (9/11), and *An. barberi* from the fewest (2/11) (Figure 4.1).

Anopheles barberi was collected mainly from NI, with one specimen obtained from a tree hole in WE, but none from OT, a region with previous records for this species, despite considerable effort to locate suitable larval habitat in this area (Figure 4.1; Table 4.5). The much greater abundance of *An. barberi* collected in NI is due to the collection method (i.e., used tires in a woodlot) employed in this region. *An. earlei* was not among the most abundant species collected, but it was collected from 8/11 regions, and from the broadest geographic range in Canada (BC, ON, RQ, and NF) (Figure 4.1). It was more common in northern regions (e.g. NO, AL, and OT), and was the only anopheline collected in RQ and NF. Interestingly, *An. earlei* was not collected in WE and only six specimens were obtained in NI (Table 4.5), which are locations that were both intensely sampled and within the known range for this species (Figure 1.9).

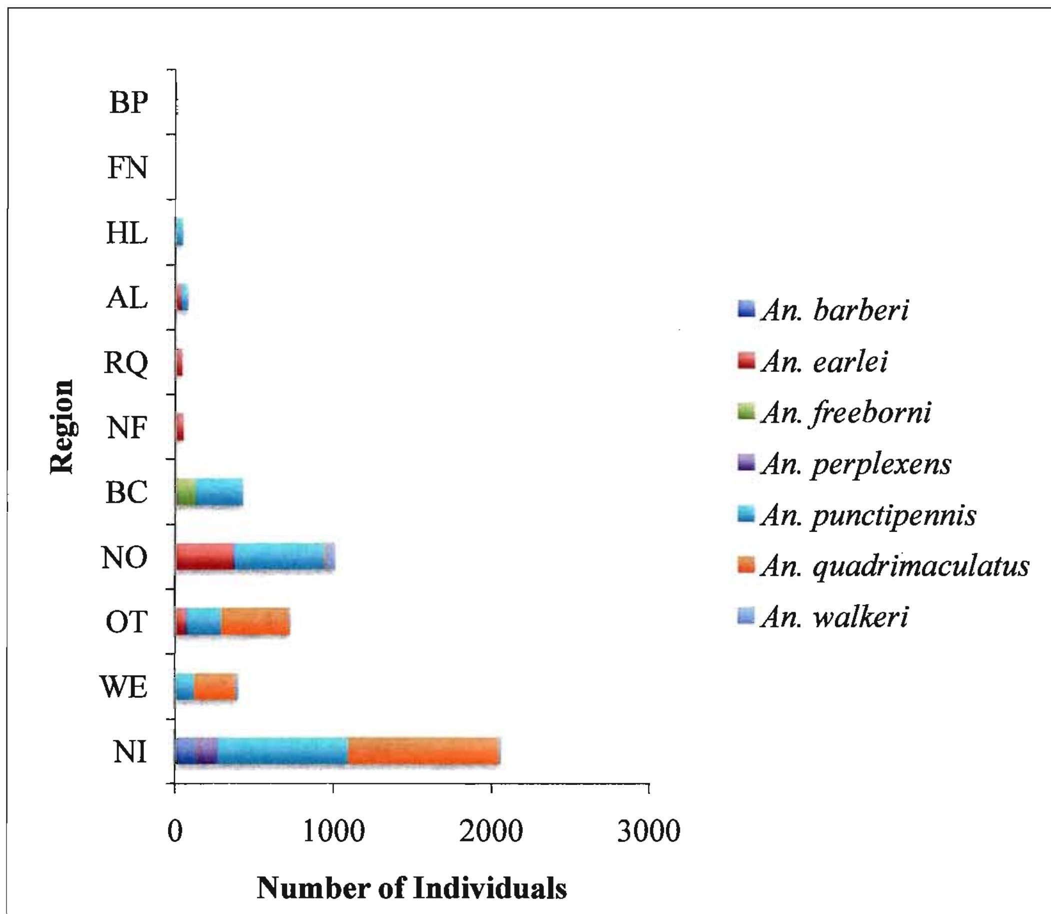

Figure 4.1. Number of *Anopheles* specimens obtained from each collecting region during 2005-2009 larval dipping collections. Region codes are listed in Appendix I.

Table 4.5. Number of specimens collected from the five main sampling regions. Region codes are listed in Appendix I.

	NI	WE	OT	NO	BC	Total
An. barberi	141	1	0	0	0	142
An. earlei	6	0	63	362	12	443
An. freeborni	0	0	0	0	114	114
An. perplexens	122	0	16	15	0	153
An. punctipennis	823	120	211	565	293	2010
An. quadrimaculatus s.l.	924	259	429	8	0	1652
An. walkeri	7	12	1	52	0	72
Total	2053	392	720	1002	419	4586

Therefore, *An. earlei* becomes more abundant, and the other anopheline species less abundant, with increasing latitude.

Anopheles freeborni was among the least commonly collected species (Figure 4.1), due in part to its presence only in the southern interior of BC (Figure 1.10). Of the three species known from BC (*An. earlei, An. freeborni*, and *An. punctipennis*), *An. freeborni* was the second most commonly collected species (Table 4.5). Unlike the most abundant species in BC (*An. punctipennis*), whose larvae are known from a wide variety water body types, the larval habitat of *An. freeborni* is restricted to clear water in open areas exposed to sunlight, such as irrigation seepages (Belton 1983). Therefore, the actual relative abundance of *An. freeborni* in nature is likely higher in regions where suitable habitat occurs.

Anopheles punctipennis was the most abundant species among all larval dipping collections, collected in the most (9/11) sampling regions of all species examined (Figure 4.1). It was absent only from RQ and NF, which are further north than the known range of this species. Within the five main collecting regions, *An. punctipennis* was the most commonly collected species in BC and NO, and the second most commonly collected species in NI, WE, and OT (Table 4.5). Due to the wide variety of habitats in which *An. punctipennis* larvae are found (Carpenter and LaCasse 1955; Wood *et al.* 1979), the high relative abundance observed for this species is likely an accurate reflection of their actual abundance within the collecting regions of this study.

Anopheles quadrimaculatus s.l. was collected only from sites in southern ON, and was the most abundant species in the three regions from which it is known (i.e., WE, NI, and OT) (Figure 4.1; Table 4.5). Although the number of specimens is very low, *An. quadrimaculatus s.l.* was also collected from NO, where it is at the northern limit of its range. For the same reasons discussed above for *An. punctipennis*, the relative abundance levels observed are likely accurate reflections of its actual abundance in these regions.

Anopheles walkeri was collected from all four sampling regions in southern ON, but in the lowest numbers of all marsh species (Figure 4.1). Interestingly, it was collected in the greatest numbers from NO (Table 4.5), the one region from which there were no previous records of this species (Wood *et al.* 1979). Furthermore, molecular data revealed the presence of a cryptic species within *An. walkeri*, with distinct isomorphic

species present in LP and NO (see Chapter Three). Because LP is well within the known range of *An. walkeri*, and *An. walkeri* larvae were collected from habitats other than the large marshes (e.g. ditches) present in WE and LP, the new cryptic species is most likely the NO species. Also, since *An. walkeri* adult females were collected in large numbers from WE using CDC light traps, but very few larvae found in nearby marshes, the low number of *An. walkeri* specimens obtained by larval dipping is likely not an accurate reflection of their actual abundance in the regions where *An. walkeri* occurs.

Since the number of specimens obtained from six of the 11 collecting regions NF, RQ, AL, HL, FN, and BP was much lower compared to five of the main regions included in the study (BC, WE, NI, OT, and NO) (Figure 4.1), the following analyses of larval habitat characteristics are based on the data from these five regions only.

4.3.3. Larval Habitat Analyses

Anopheline larvae were collected from a wide variety of water body types, from small stagnant ponds to the slowly-flowing water of lake and river edges (types and definitions as used in this thesis listed in Table 4.2). Due to similarity between certain habitat types, some were grouped together for the remainder of analyses (e.g., marshes and shallow waters). The number of specimens obtained varied depending on the species and the water body type from which they were collected (Table 4.6). Anopheline larvae were most commonly collected from ditches and creeks (38.0%), followed by ponds (20.1%), marshes and shallow waters (12.3%), and least commonly from artificial containers and tires (5.6%), forest and ground pools (1.6%), and tree holes (0.2%) (Table 4.6).

Anopheles barberi was collected from only two water body types and two regions in Ontario. Except for one specimen from a tree hole at Point Pelee National Park (WE) and 8 from tree holes in NI, *An. barberi* was collected mainly from artificial containers (tires) in Niagara (Table 4.6; Figure 4.2). This is due to repeated trips to this location to obtain specimens, illustrating the advantage of using this particular method (i.e., used tires in a woodlot) over locating tree holes that contain *An. barberi* larvae.

Table 4.6. Number of *Anopheles* specimens collected from each water body type in five main collecting regions during 2005-2009 field seasons (in descending order of abundance).

	barb	earl	free	perp	punc	quad	walk	Total
ditches/creeks	0	105	4	78	944	597	13	1741
ponds	0	37	110	9	380	384	4	924
marshes/shallow waters	0	120	0	5	202	211	24	562
lake/river edges	0	25	0	7	133	239	7	411
swamps/sloughs	0	119	0	8	143	61	24	355
alvar/quarry	0	3	0	18	82	153	0	256
artificial/tires	133	0	0	28	94	0	0	255
forest/ground pools	0	34	0	0	32	7	0	73
tree holes	9	0	0	0	0	0	0	9
bogs	0	0	0	0	0	0	0	0
Total	142	443	114	153	2010	1652	72	4586

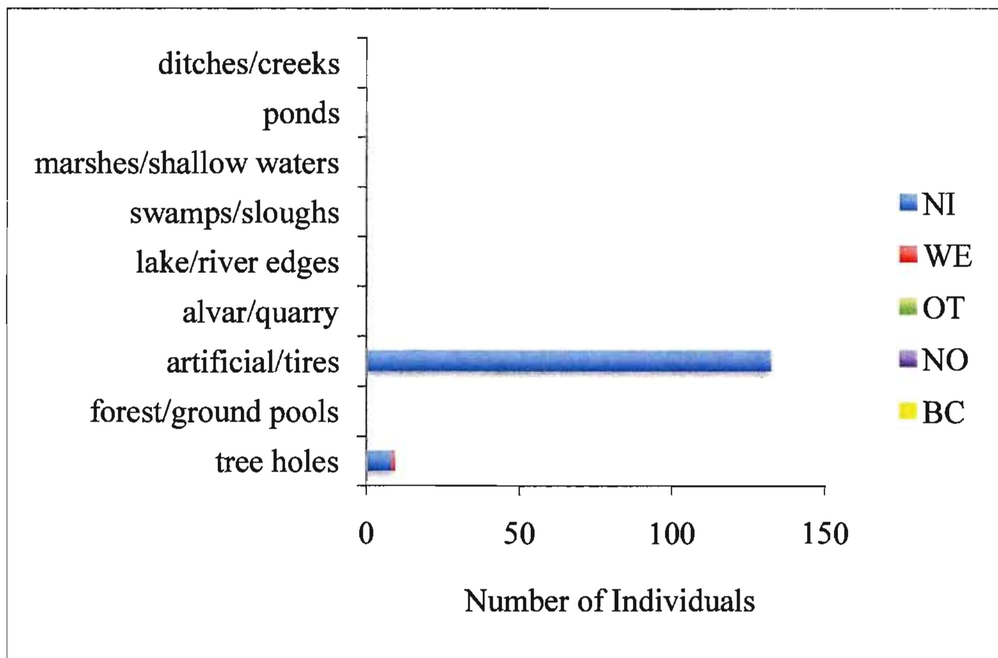

Figure 4.2. Number of *An. barberi* larvae collected from each larval habitat type. Definitions listed in Table 4.2; Region codes listed in Appendix I.

Anopheles freeborni was the only species collected from even fewer water body types and regions than *An. barberi*. *Anopheles freeborni* was collected only in British Columbia, mainly from ponds, but also in creeks and ditches (Table 4.6; Figure 4.3). During the 10-day collecting trip in BC, suitable larval habitat was not easy to find, but *Anopheles* were found in almost all ponds checked, most of which were located on golf courses and farms. *Anopheles freeborni* were obtained from 8/27 BC larval dipping collections, but most specimens (79.8%) came from 2 collections made on the same farm near Kamloops at different times. Therefore, the limited number of water body types in which *An. freeborni* larvae were found in this study may not represent all potential habitats within its distribution range. For example, *An. freeborni* are known from clear water in sunlit areas (Belton 1983), such as irrigation ditches, only one of which was sampled while collecting near Oliver, BC.

Anopheles earlei was collected from a wide variety of habitats. It was most commonly collected from marshes and shallow waters (27.1%), swamps and sloughs (26.9%), and ditches and creeks (23.7%) (Table 4.6; Figure 4.4). *Anopheles earlei* was collected from the greatest number of larval habitat types within the region in which it was most often collected (i.e., NO). As latitude decreased, so too did the relative abundance of *An. earlei*, as well as the number of larval habitat types from which it was collected. Also, as most *An. earlei* specimens were collected from habitats located in more pristine areas (A. Thielman, *pers. obs.*), the greater urban development in NI and WE may also contribute to the low number of specimens collected in these regions.

Anopheles punctipennis was collected in all five main regions, and from all possible larval habitats sampled except tree holes (Table 4.6; Figure 4.5). The most common habitat types in which *An. punctipennis* were found were ditches and creeks (47.0%), followed by ponds (18.9%), and marshes and swamps (10.0%). *Anopheles punctipennis* was collected from the greatest number of larval habitats types in NI, followed by NO, WE, and OT, and the least in BC. However, these values may reflect the additional sampling effort in NI (Figure 4.1). *Anopheles punctipennis* (including those identified as *An. perplexens*) was the only anopheline except *An. barberi* that was found in artificial containers (i.e., tires).

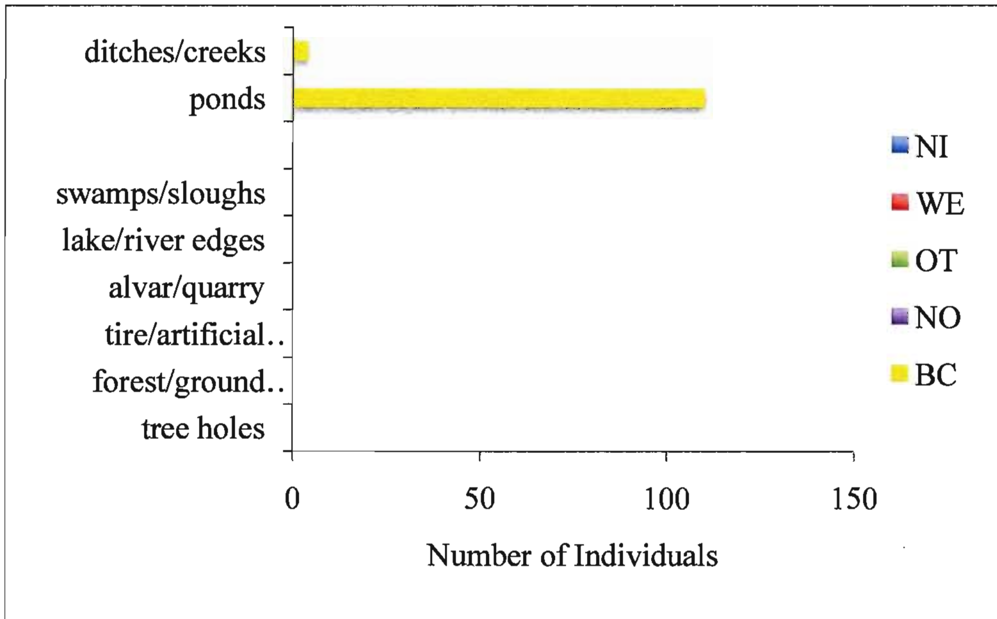

Figure 4.3. Number of *An. freeborni* larvae collected from each larval habitat type. Definitions listed in Table 4.2; region codes listed in Appendix I.

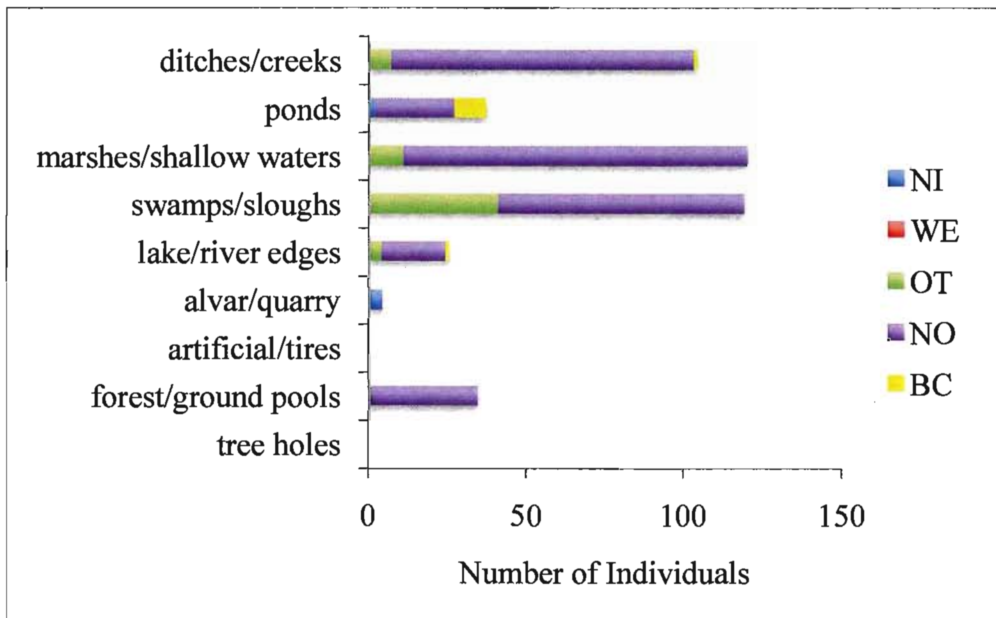

Figure 4.4. Number of *An. earlei* larvae collected from each larval habitat type. Definitions listed in Table 4.2; region codes listed in Appendix I.

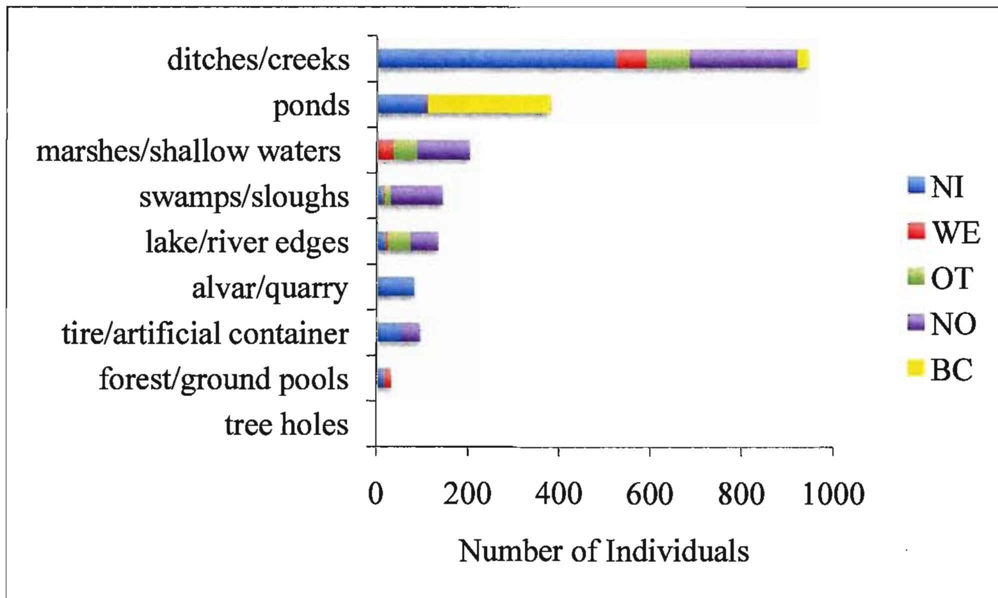

Figure 4.5. Number of *An. punctipennis* larvae collected from each larval habitat type. Definitions listed in Table 4.2; region codes listed in Appendix I.

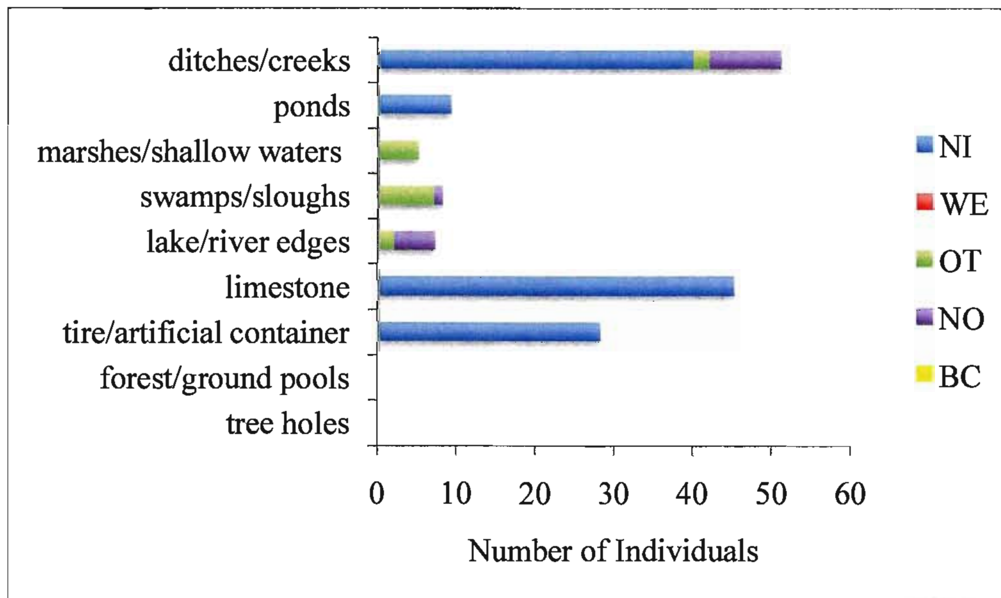

Figure 4.6. Number of *An. perplexens* larvae collected from each larval habitat type. Definitions listed in Table 4.2; region codes listed in Appendix I.

Specimens identified as suspected cryptic species *An. perplexens* were collected mainly from NI, but also from sites in NO and OT (Table 4.6; Figure 4.6). In NI, most *An. perplexens* specimens were collected from limestone habitats (36.9%) and ditches and creeks (32.8%), and artificial containers (i.e., tires) (23.0%). In NO and OT, *An. perplexens* was also collected from ditches and creeks (35.5%), but also from swamps and sloughs (25.8%), lake and river edges (22.6%). Within its known range, the larvae of suspected cryptic species *An. perplexens* is restricted to alvar habitats.

Larval dipping sites were classified as a limestone habitat (ditch/creek, alvar, or quarry), if appropriate, in the Niagara region only (Figure 4.7). The presence of alvars in NO and OT were unknown during collecting trips to these regions, and reclassification of each larval dipping site afterwards as one of the limestone types of habitats was not possible. In addition to known alvars in WE and OT (Reschke *et al.* 1999), habitats with exposed limestone were discovered in NI (Figure 4.7). Alvars were also located, including a man-made alvar (i.e., topsoil removed decades ago from a property exposing limestone) known as Marcy's Woods in Fort Erie, the second was a shagbark-hickory alvar located between a new residential area and the Wainfleet Wetlands in Port Colbourne, and the third was a red cedar alvar located on a quarry property in Wainfleet.

Interestingly, the three main regions from which *An. perplexens* specimens were collected are the same three regions from which alvars are known, i.e., NO, OT, and NI (Figure 4.8). However, unlike in the type locality for *An. perplexens*, the larval habitats of *An. punctipennis* and *An. perplexens* (Figures 4.5 and 4.6) are not distinctly different, sharing many of the same types of habitats. Therefore, despite an apparent ecological association between *An. perplexens* specimens and alvar-type habitats in Ontario, ecological data could not confirm the presence of *An. perplexens* in this region.

Anopheles quadrimaculatus s.l. was the second most commonly collected species overall, but was the most common species in the three main regions in which it occurs (WE, NI, and OT) (Table 4.6; Figure 4.9). The distribution of *An. quadrimaculatus s.l.* approaches NO, but only 8 specimens were collected there despite extensive sampling in the region. It was also collected from the second widest variety of larval habitat types (7/9). *Anopheles quadrimaculatus s.l.* was most commonly collected from ditches and creeks (36.2%), and ponds (23.2%), and lake and river edges (14.4%). Dominant larval

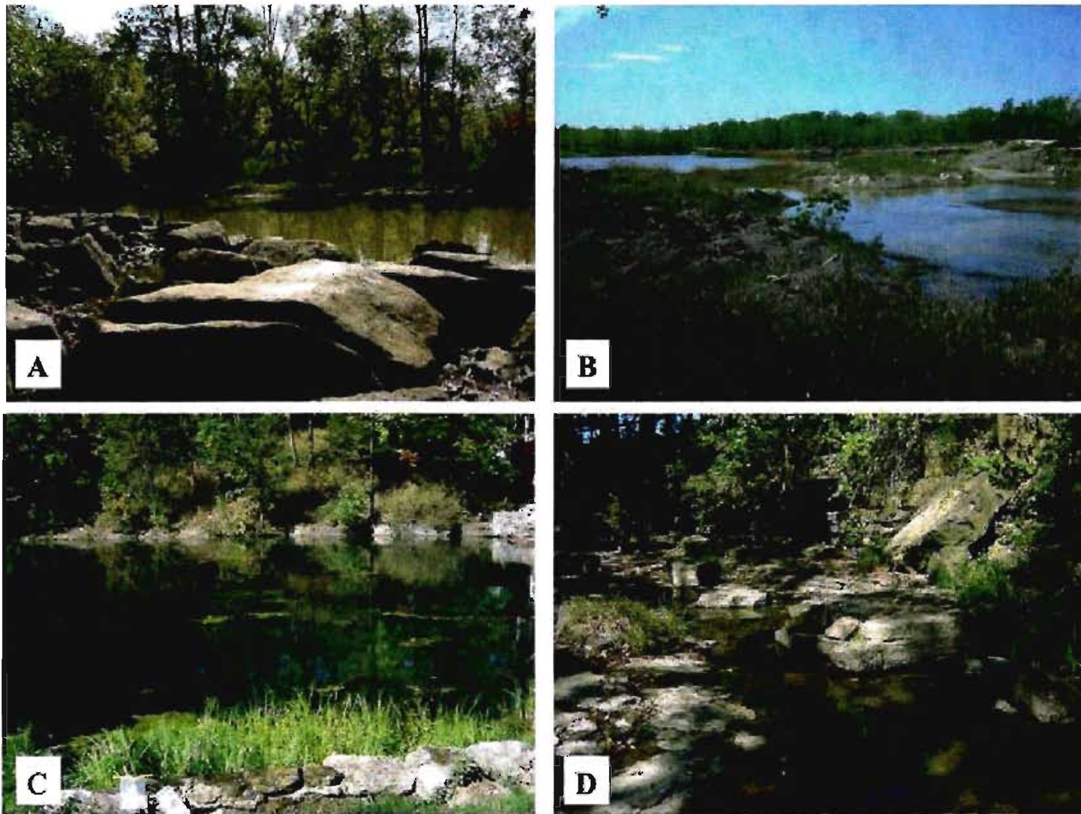

Figure 4.7. Larval habitats in the Niagara region with exposed limestone.
A - muddy creek in Effingham with small pools of clear water along edges
B - large quarry ponds in Queenston
C - pooled water above a limestone waterfall
D - clear, rapidly flowing creek in Effingham with layers of limestone visible

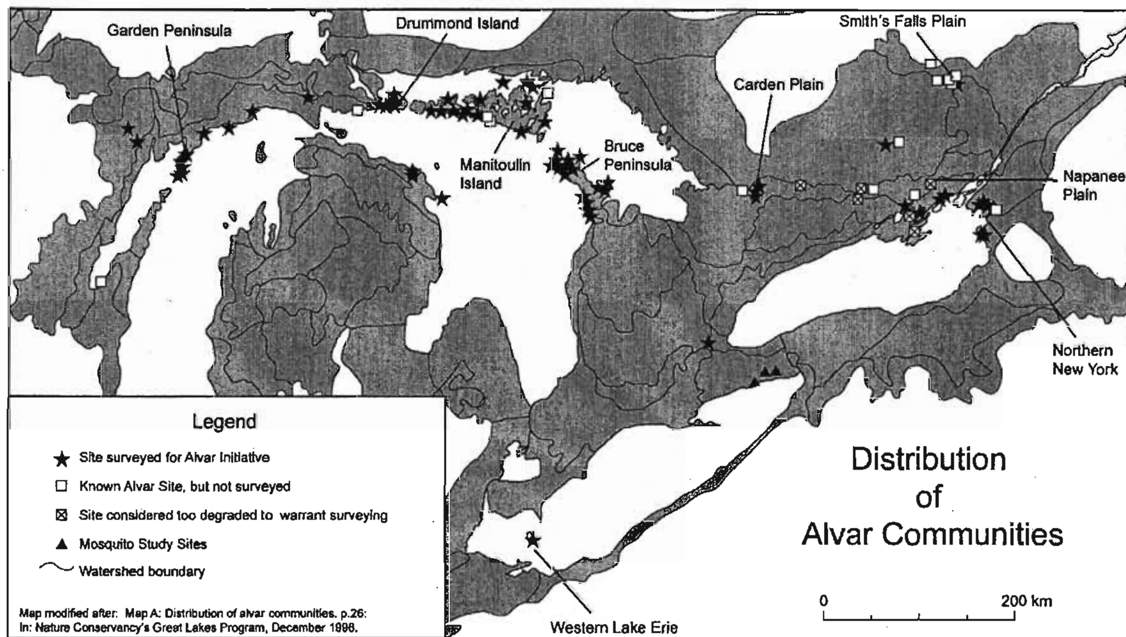

Figure 4.8. Map of alvar communities present in southern Ontario (Map A from Reschke *et al.* 1999). Black stars and white squares indicate known alvar sites (Reschke *et al.* 1999) and triangles indicate alvar sites discovered in Niagara through the current study. In addition to alvar sites, many old limestone quarries were also located and sampled during the course of this study.

habitat type varied depending on region, with ditches and creeks being the most common type in NI and WE, and lake and river edges in OT (Figure 4.9).

Although *An. quadrimaculatus s.l.* is at the edge of its range in southern Ontario and Quebec, it was the most abundant species collected in the WE, NI, and OT regions. This species was common in roadside ditches, possibly due to an increased ability to tolerate saline conditions in the larval habitat (Wood *et al.* 1979). It appears to be more common in developed areas and less common in pristine ones (A. Thielman, *pers. obs.*). Since *An. quadrimaculatus s.l.* is a known vector of malaria parasites in North America (Wood *et al.* 1979), and is common in densely populated regions from WE to OT, effort to control this species in southern Ontario should be of primary concern if autochthonous transmission of this disease were to reemerge in the future.

Anopheles walkeri was the least commonly collected species, present in relatively low numbers in four regions, and collected from five larval habitat types (Table 4.6; Figure 4.10). It was collected most often from marshes and shallow water (33.3%), and swamps and sloughs (33.3%), but also from ditches and creeks (18.0%). *Anopheles walkeri* was collected least often from OT, with only one specimen collected from a lake/river edge. In NI, three specimens were collected from ditches and creeks and four from ponds. In WE, four *An. walkeri* larvae were obtained from ditches and creeks and eight from marshes/shallow waters (Figure 4.10).

As most *An. walkeri* larvae (72%) were collected from NO, a region from which there are no previous records, and molecular data suggested the possible presence of a cryptic species within *An. walkeri*, further studies of *An. walkeri* from NO are warranted. Dominant larval habitat varied depending on the region, with ponds being the most common type in NI, marshes and shallow waters in WE, and swamps and sloughs in NO (Figure 4.10). Thus, although differences in the larval habitats of each cryptic species were not apparent, geographical data confirm the presence of a cryptic species within *An. walkeri* that is specific to NO.

Overall, the main larval habitat type from which anopheline larvae were collected was ditches and creeks, with almost triple the specimens obtained than from marshes and shallow waters (37% vs. 12.3%) (Table 4.6). So, while all four "marsh" species

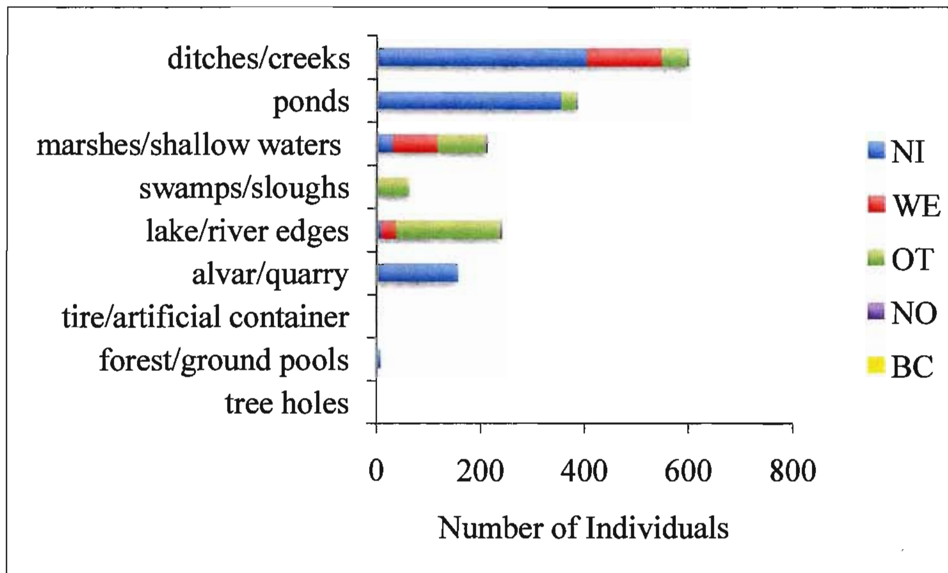

Figure 4.9. Number of *An. quadrimaculatus s.l.* larvae collected from each larval habitat type. Definitions listed in Table 4.2; region codes listed in Appendix I.

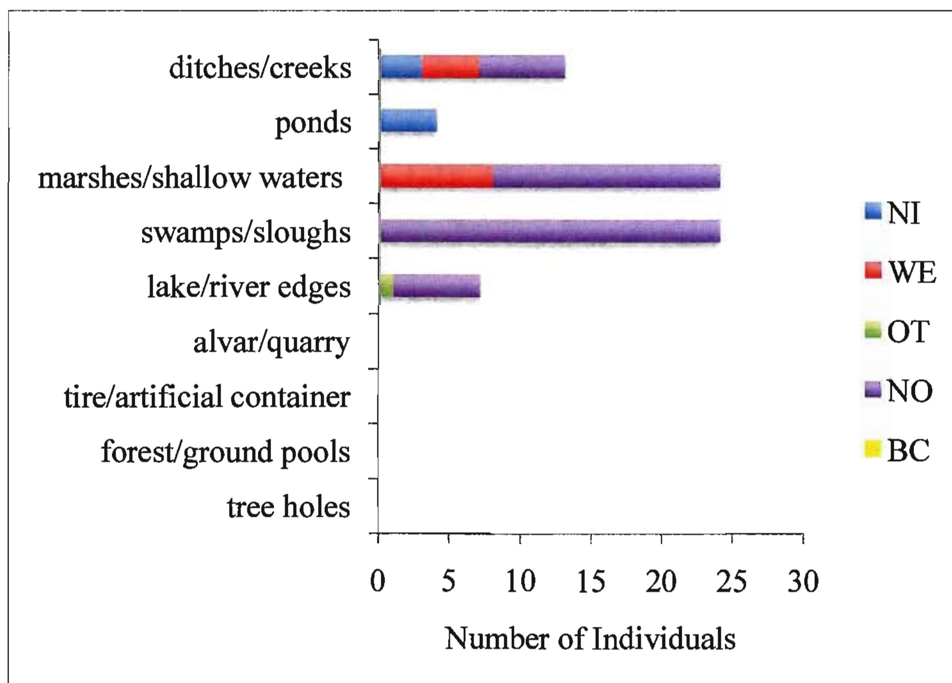

Figure 4.10. Number of *An. walkeri* larvae collected from each larval habitat type. Definitions listed in Table 4.2; region codes listed in Appendix I.

(i.e., *An. earlei, An. punctipennis, An. quadrimaculatus,* and *An. walkeri*) were found in this habitat, it was not the dominant larval habitat type associated with these species. Large marshes such as the ones at Point Pelee National Park and Provincial Parks located along the north shores of Lake Erie (Rondeau, Long Point, etc.) likely produce extremely large populations of *Anopheles* mosquitoes throughout the entire mosquito season. However, many marshes in Ontario have been drained for agricultural or other land uses. Therefore, the greater relative abundance of anophelines collected from ditches could be due to a decrease in marsh habitat, and an increase in the number of roads, and therefore ditches, in these areas. All species examined in this study, except *An. barberi*, were collected from ditches and creeks.

The second most common water body type associated with *Anopheles* larvae was ponds (20.1% of all specimens collected), and all species, except *An. barberi,* were collected from this habitat (Table 4.6). The next most common *Anopheles*-positive larval habitat types were marshes/shallow waters (12.3%), swamps/sloughs (7.7%), and lake/river edges (8.9%). These larval habitats are all relatively large in size compared to other habitats types, with emergent and floating vegetation present providing protection from water movement. All four "marsh" species were collected from these habitats in relatively similar proportions.

Only two species were collected from tires, *An. barberi* and *An. punctipennis* (including those identified morphologically as *An. perplexens*), comprising only 5.6% of all anophelines collected (Table 4.6). However, this particular habitat was not encountered often in nature, and almost all specimens were obtained from multiple visits to the same set of tires placed in the Niagara woodlot to collect *An. barberi.* Approximately the same amount (5.6%) was collected from alvars and quarries, habitats intentionally sought during 2007-2009 field seasons for egg morphology studies (see Chapter Two). The lack of anopheline specimens collected from forest and ground pools (1.6%) reflects in part the relatively lower number of forest or ground pools sampled combined as well as an actual absence from many such habitats that were sampled (A. Thielman, *pers. obs.*).

Very rarely, anophelines were collected from bogs, a habitat characterized by acidic waters and distinct vegetation such as peat and sedges, which were only found and

sampled in BP and RQ. *Anopheles earlei* was found in the greatest number (n=36), but a few specimens of the other marsh anophelines were also collected. In Canada, bogs are more common in northern regions, and were common in RQ and NF, two regions where only *An. earlei* occur. The ability of *An. earlei* to tolerate the acidity associated with bogs might contribute to this species' extensive range in Canada, occurring farther north than any other anopheline, present even in boreal regions of the Yukon (and Alaska) (Figure 1.9).

Finally, the water body type from which the fewest number of *Anopheles* larvae was obtained was tree holes, the primary larval habitat for *An. barberi.* Only nine specimens, or 0.2% of all those collected, came from tree holes, despite much effort to locate tree holes in regions where *An. barberi* had been previously recorded, and sampling of the water found in many tree holes that were discovered.

4.3.4. Emergent and Floating Vegetation

Studies of the larval habitat of *Anopheles* mosquitoes often includes analyses of both emergent and floating vegetation types, as species-vegetation associations can aid in the location of suitable larval habitat for vector control in areas where disease transmission occurs (Rejmankova *et al.* 1993; Manguin *et al.* 1996; Gimnig *et al.* 2001; Shililu *et al.* 2003). The presence or absence of emergent (EV) and floating (FV) vegetation (and types if present) was recorded for each larval dipping collection (type listed in Table 4.3). Therefore, for each larval dipping collection, four possible vegetation categories were constructed: both EV and FV absent; EV absent and FV present; EV present and FV absent; and both EV and FV present.

Of the 350 larval dipping collections conducted during the 2005-2009 field seasons for which ecological data were recorded, *Anopheles* larvae were present in 224 (63.1%). Although ecological data from the remaining *Anopheles*-negative 126 larval dipping collection were recorded, data from sites in which no mosquitoes of any species were collected was *not* recorded. Therefore, only analysis of vegetation data from the *Anopheles*-positive sites was possible, and three collections were omitted from the

following analyses due to questionable species identifications that could not be confirmed.

Among the 221 *Anopheles*-positive larval dipping collections examined, *Anopheles* larvae were collected most often from habitats with both FV and EV present (53.8%), with much lower proportions collected when FV was present and EV absent (18.1%), when FV absent and EV present (14.9%), and when both FV and EV were absent (13.1%) (Figure 4.11). Combining vegetation types, a much greater proportion of *Anopheles* larvae were collected from habitats in which at least one type of vegetation was present (86.9%) compared to habitats in which both were absent. Within the *Anopheles*-negative larval dipping collections (n=129) for which ecological were recorded, FV and EV were present in 33.4% of them, and 60.3% of them had at least one type of vegetation present. Therefore, this confirms anecdotal descriptions by early authors that anophelines are usually associated with floating or emergent vegetation (Carpenter and LaCasse 1955; Wood *et al.* 1979).

Presence or absence of emergent and floating vegetation in the larval habitat was also analysed for each species separately, to determine if the anophelines examined were associated with a particular vegetation type category. For each species, the relative proportion of vegetation categories associated with each species was determined (Figure 4.12).

Most distinct in larval habitat association is *An. barberi,* with all collections from larval habitats in which both EV and FV were absent (n=14), as expected for tree holes and tires (Figure 4.12). *Anopheles freeborni* was collected in similar proportions from larval habitats in which at least one type of vegetation was present (27.3%-36.4%), and never from habitats in which both types were absent. The remaining marsh species (i.e., *An. freeborni, An. punctipennis, An. quadrimaculatus s.l.,* and *An. walkeri*) exhibited similar patterns in the relative proportions of vegetation categories associated with each species, but, unlike *An. freeborni*, all were also collected from habitats in which FV and EV were both absent (Figure 4.12).

All four remaining species examined were collected in the greatest proportion when both EV and FV were present in the larval habitat, from 50% in *An. walkeri* to 66.7% in *An. quadrimaculatus s.l.* (Figure 4.12). Combining vegetation types, they were

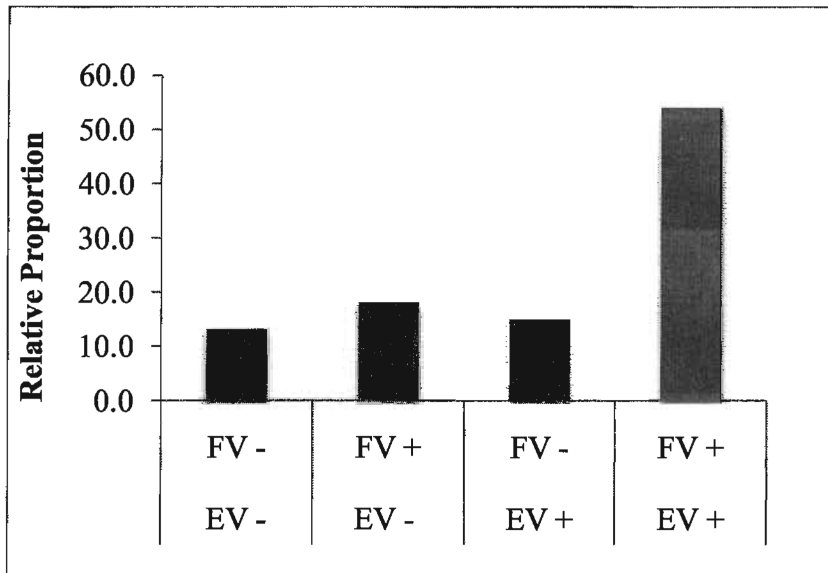

Figure 4.11. Relative proportion of vegetation type categories recorded from *Anopheles*-positive larval dipping collections. EV = emergent vegetation; FV = floating vegetation; - = absent; + = present.

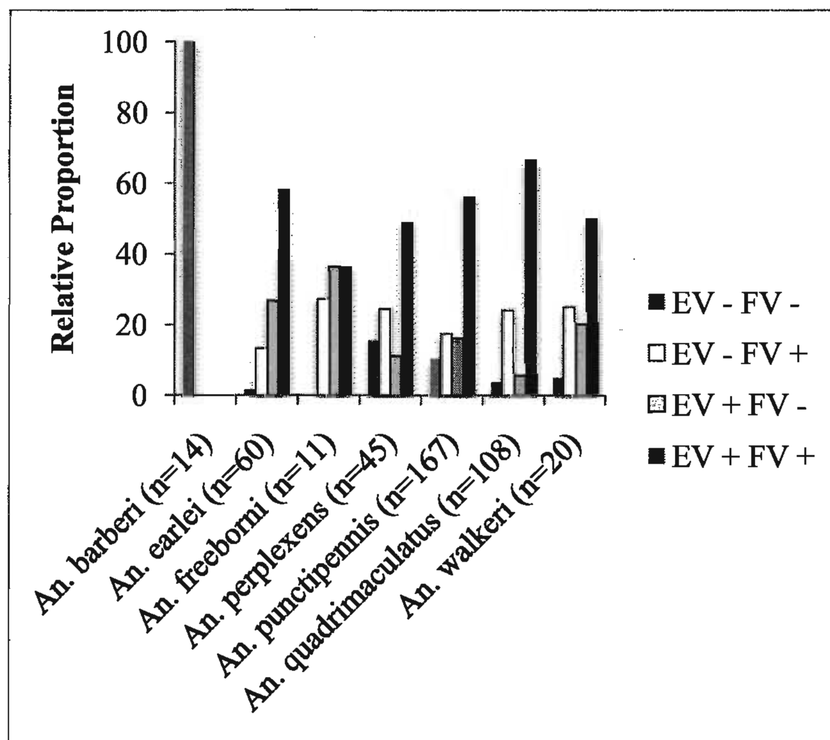

Figure 4.12. Relative proportion of vegetation categories associated with each species. EV = emergent vegetation; FV = floating vegetation; - = absent; + = present.

present in ~85% to 98% of collections when at least one vegetation type was present. *Anopheles earlei* larvae were collected in the second greatest proportion when EV was present and FV absent (26.7%), followed by EV absent and FV present (13.3%), only rarely when both are absent (1.7%). Therefore, EV is likely a better indicator than FV of potential *An. earlei* larval habitats. *An. punctipennis* larvae were collected in the second greatest proportion when FV was present and EV absent (17.4%), followed FV absent and EV present (16.2%), and when both are absent (11.1%). A very similar pattern was observed for specimens identified as *An. perplexens*.

Larvae of *An. quadrimaculatus s.l.* were collected in the second greatest proportion when only FV was present (24.1%), but considerably fewer specimens were obtained from habitats with only EV present (5.6%) or both types absent (3.7%). The pattern observed for *An. walkeri* is very similar to that of *An. punctipennis*, occurring in the second greatest proportion when FV was present and EV absent (25%), followed by when FV absent and EV present (20%), and rarely when both are absent (5%). Therefore, unlike *An. earlei*, FV is likely a better indicator of potential *An. punctipennis*, *An. quadrimaculatus s.l.*, and *An. walkeri* larval habitats than EV, particularly for *An. quadrimaculatus s.l.* with ~90% of all specimens collected from habitats in which FV was present (Figure 4.12).

In addition to the presence of FV and EV in the larval habitat, the types of FV and EV (listed in Table 4.3) present in the larval habitats of the anophelines examined were also analysed. Cattails and grasses were the most common types of EV recorded, present in 40% and 38% of all *Anopheles*-positive collections, respectively, and terrestrial plants present in 8.6% (Figure 4.13). Other types of EV were present in *Anopheles*-positive larval habitats (such as shrubs, common reeds, sword plant, and wood), but were in less than 10 larval dipping collections and were therefore omitted from Figure 4.13.

Many types of FV were also recorded from *Anopheles*-positive larval habitats, and more types than EV were found in 10 or more larval dipping collections (Figure 4.14). Algae was the most common FV type, found in 51% of *Anopheles*-positive collections, and was the dominant type found with all species except *An. walkeri*, in which it was co-dominant with duckweed, both present in 50% of the *An. walkeri*-positive larval dipping collections (n=20). Duckweed was the second most common FV

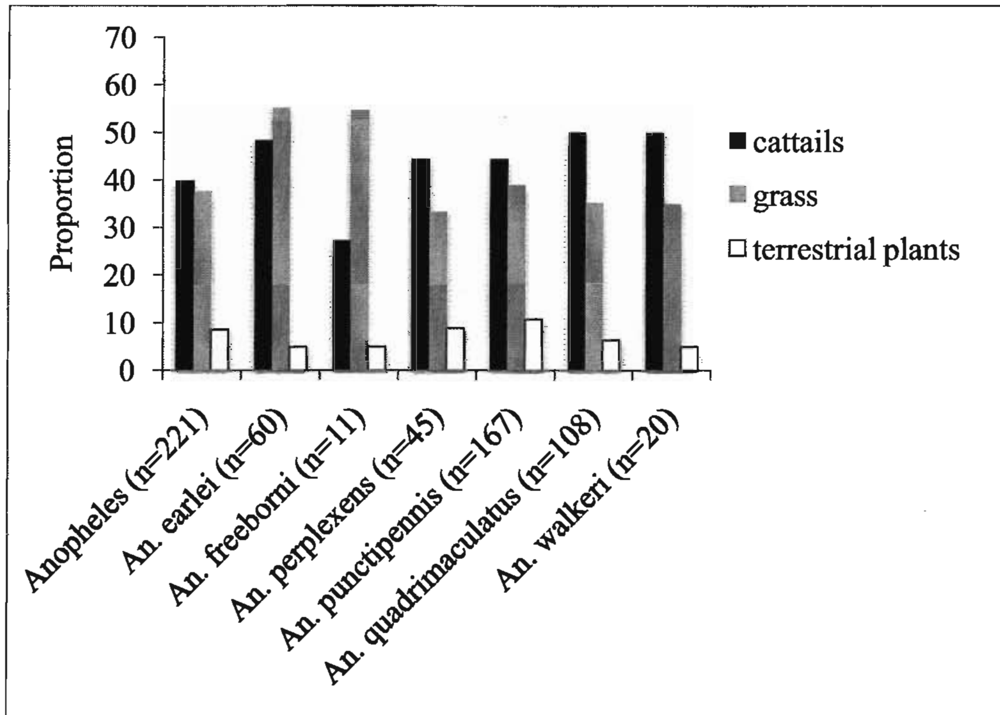

Figure 4.13. Proportion of larval dipping collections in which each of the dominant types of emergent vegetation was present for each species separately and for all *Anopheles* larvae combined.

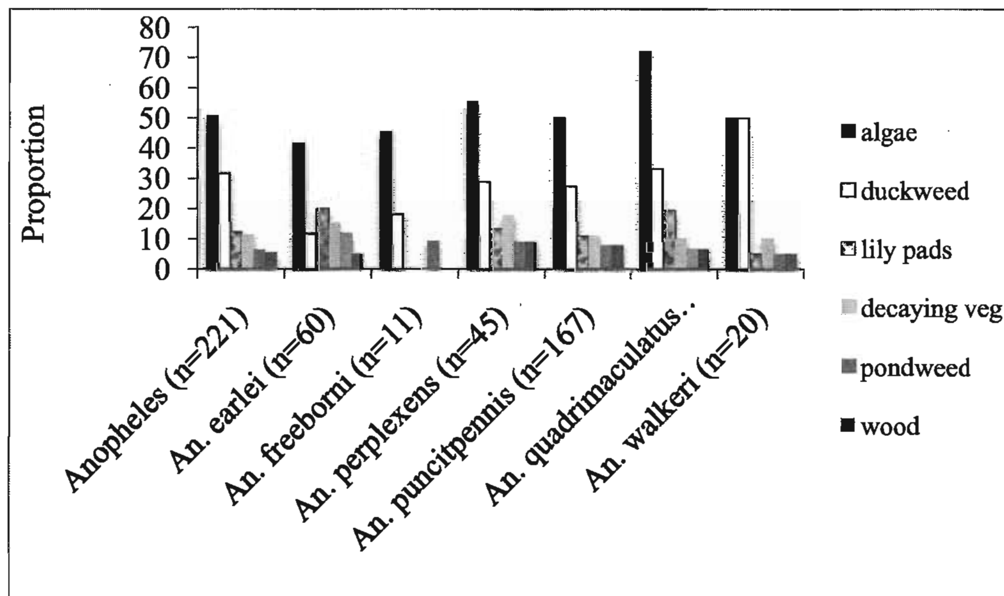

Figure 4.14. Proportion of larval dipping collections in which each of the dominant types of floating vegetation was present for each species separately and for all *Anopheles* larvae combined.

type in *Anopheles*-positive collections at 31%, and in those of all species except *An. walkeri* (as mentioned above) and *An. earlei*. The second most common FV type in *An. earlei* was lily pads, which were present in 20% of *An. earlei*-positive collections, as well as the larval dipping collections of *An. quadrimaculatus s.l.* (19.4%), *An. perplexens* (13.3%), *An. punctipennis* (10.8%), and *An. walkeri* (5.0%). Decaying vegetation was found in about 10% of *Anopheles*-positive collections, and in those of all species examined (except *An. freeborni*), from 10% in *An. walkeri* to 17.8% in *An. perplexens*. Pondweed was found in 5.0% to 11.7% of the larval dipping collections and wood from 5.0% to 9.0% of those from all species except *An. freeborni* (0%). Other FV types recorded from *Anopheles*-positive larval dipping collections (n<10) include grass, wood, pondweed, moss and lichen.

4.3.5. Species Associations

As with the presence of floating and emergent vegetation, early authors often state that certain anopheline species are usually in association with the other marsh species (Wood *et al.* 1979), but quantitative studies of species association have not been done for *Anopheles* from Canada. Therefore, larval dipping collection data were analysed to determine species associations for the anophelines examined. *Anopheles barberi* was the species most often collected alone, with 71.4% of *An. barberi*-positive larval dipping collections (n=14) containing only *An. barberi* larvae (Table 4.7). However, it was found with *An. punctipennis* in 28.6% of *An. barberi*-positive collections, including those identified as *An. perplexens* in 21.4%. There were two species that were never the only species obtained in a larval dipping collection, *An. perplexens* and *An. walkeri* (Table 4.7). *Anopheles walkeri* was found most often with *An. punctipennis* (co-occur in 80% of *An. walkeri*-positive collections), followed by *An. quadrimaculatus* (60%), and *An. earlei* (35%) (but never with *An. freeborni* as their distribution ranges do not overlap).

With respect to specimens identified morphologically as *An. perplexens*, larvae were found with *An. barberi* and *An. walkeri* in 6.7% of larval dipping collections form which *An. perplexens*-type females were obtained, with *An. earlei* in 20.0%, and with *An. quadrimaculatus s.l.* in 55.6%. Most notably, females identified as *An. perplexens* (based

Table 4.7. Relative frequencies of species co-occurrences among the anopheline species collected in *Anopheles*-positive larval dipping collections (n=221). For each species along the top row, the proportions of all other species present in the larval dipping collections positive for that species are shown in the column below.

	An. barberi (n=14)	*An. earlei* (n=60)	*An. freeborni* (n=11)	*An. perplexens* (n=45)	*An. punctipennis* (n=169)	*An. quadrimaculatus* (n=108)	*An. walkeri* (n=20)
An. barberi	**71.4**	0.0	0.0	6.7	2.4	0.0	0.0
An. earlei	0.0	**23.3**	45.5	20.0	23.7	17.4	35.0
An. freeborni	0.0	8.3	**27.3**	0.0	4.1	0.0	0.0
An. perplexens	21.4	15.0	0.0	**0.0**	26.6	22.9	15.0
An. punctipennis	28.6	66.7	63.6	100.0	**20.7**	78.0	80.0
An. quadrimaculatus s.l.	0.0	31.7	0.0	55.6	50.3	**14.7**	60.0
An. walkeri	0.0	11.7	0.0	6.7	9.5	11.0	**0.0**

on morphology) were associated with *An. punctipennis* in 100% of the collections that were positive for *An. perplexens*-type individuals. Since *An. perplexens* and *An. punctipennis* are known to occupy different larval habitats in the type locality for *An. perplexens*, this is further evidence that the specimens identified morphologically as *An. perplexens* likely represent morphological variants of *An. punctipennis* near the northern limits of this species range.

The distribution ranges of *An. freeborni* and *An. barberi*, *An. quadrimaculatus s.s.* or *An. walkeri* do not overlap and, thus, these species were never collected together (Table 4.7). However, in BC where *An. freeborni* occurs, it was found more often in association with the other species known from this region, i.e., *An. punctipennis* (63.6%) and *An. earlei* (45.5%), than it was found alone (27.3% of collections).

Anopheles earlei was the only species obtained in 23.3% of *An. earlei*-positive collections, and therefore associated with at least one of the other anophelines in ~77% of them (Table 4.7). *Anopheles earlei* was present with *An. freeborni* in 8.3% of *An. earlei*-positive collections, with *An. walkeri* in 11.7% of them, with *An. quadrimaculatus s.l.* in 31.7%, and with *An. punctipennis* in 66.7% of them. Therefore, both *An. earlei* and *An. freeborni* were collected most often with *An. punctipennis* (i.e. 66.7% and 63.6% of *An. earlei*-positive and *An. freeborni*-positive larval dipping collections, respectively).

Anopheles quadrimaculatus s.l. was collected alone in 14.7% of *An. quadrimaculatus s.l.*-positive collections, and therefore with at least one other anopheline 85.3% of *An-quadrimaculatus s.l.*-positive larval dipping collections (Table 4.7). *Anopheles quadrimaculatus s.l.* was found with *An. walkeri* (11.0%), *An. earlei* (17.4%), and most often with *An. punctipennis* (78%) of *An. quadrimaculatus*-positive collections.

Finally, *An. punctipennis,* the species collected most often, from the widest variety of larval habitat types and in association with the widest variety of emergent and floating vegetation types, was found most often associated with at least one of the other species (Table 4.7). While collected alone in 20.7% of *An. punctipennis*-positive larval dipping collections, it was also collected with every other species that occurs in Canada (except *An. crucians s.l.* as this species was not collected during the course of this study), i.e., *An. barberi* (2.4%), *An. freeborni* (4.1%), *An. walkeri* (9.5%), *An. earlei* (23.7%), and *An. quadrimaculatus* (50.3%).

Therefore, while all species, except *An. walkeri* and those identified as *An. perplexens*, were sometimes found alone in larval dipping collections, they were usually associated (more than 70% of the time) with at least one of the other anopheline species included in the study. Overall, *An. earlei* was collected with *An. freeborni* or the other marsh species in 76.7% of collections, *An. freeborni* with the other two species that share its range in 72.7% of collections, *An. punctipennis* with any of the other anophelines in 79.3% of collections, and *An. walkeri* and *An. quadrimaculatus s.l.* with the other marsh species in 100% and 85.3% of larval dipping collections, respectively.

4.4. Summary

During the 2005-06 field seasons, sampling effort was approximately equal in four of the five main collecting regions (BC, WE, OT, and NO) (Figure 4.1). However, because the overall objective of this study was to determine whether cryptic species were present among the *Anopheles* species of Canada, collection effort was focused in certain areas during the 2007-2009 field seasons, particularly the fifth main collecting region (i.e., NI) due to the potential association between alvar-type habitats and *An. perplexens*-type specimens in this region. Therefore, the relative abundances of each species collected that were observed in this study are likely due in part to differences in sampling intensity conducted in each region. In addition, the types of water bodies from which larvae were collected were influenced by their ease of location. As time is a limited resource on collecting trips, larval dipping sites were often selected based on their proximity to roads and trails to decrease the amount of time spent at each location and maximize the number of collections (and specimens collected) in each region. Therefore, while the ecological data obtained during the course of the study may not accurately reflect species' abundances in certain regions or water body types in nature, it represents the most detailed information regarding larval habitats associated with *Anopheles* mosquitoes in Canada.

Anopheles barberi was one of the least common species collected, found mainly in tree holes and tires in the Niagara, despite effort to locate tree holes in regions where previous specimens were known. It was never associated with any type of vegetation,

due to nature of the larval habitat, and was often collected alone, although it was found with *An. punctipennis* when collected from tires. This species is likely present throughout southern Ontario from Point Pelee National Park to Ottawa, wherever suitable habitat (i.e., hardwood forests) exists. However, they are not commonly collected through routine surveillance (A. Thielman, *pers. obs.*) and suitable tree holes can be extremely difficult to locate or reach without tree-climbing or other equipment.

Anopheles earlei was collected from the most geographically distant locations, including British Columbia, throughout southern Ontario, Radisson in northern Quebec, and the island of Newfoundland, for which it is a first record. It was the third most common species collected, comprising 11.7% of the 4805 specimens obtained through larval dipping collections. Most *An. earlei* larvae were collected from Manitoulin Island (82%), followed by Ottawa (15%), British Columbia (3%), and Niagara (1%), and none collected in the Windsor region despite being well within its known range. It was found most often in swamps, marshes, ditches and creeks, but in a variety of other habitats as well, including forest pools which is likely a common larval habitat for this species in northern wooded areas where it is common (Wood *et al.* 1979). *Anopheles earlei* larvae were collected most often when both emergent and floating vegetation were present, sometimes when only one type was present, and rarely when both were absent. Vegetation types with which it was commonly found include grasses, cattails, algae, and duckweed. *Anopheles earlei* was sometimes the only species obtained in larval dipping collections (23%), but was more often present with one or more of the other species, i.e., *An. punctipennis* in 67%, *An. quadrimaculatus s.l.* in 32%, *An. walkeri* in 11%, and *An. freeborni* in 8% of all *An. earlei*-positive larval dipping collections.

Anopheles freeborni was collected in smaller numbers, comprising 2.4% of all specimens obtained, due in part to the low number of larval dipping collections conducted in BC and their absence from the Lower Fraser Valley where some of the larval dipping collections were conducted. It was collected mainly from ponds located on farms and golf courses, but from a few ditches and creeks as well. *Anopheles freeborni* larvae were always found in habitats with either emergent or floating vegetation present, the most common types of which were grasses and cattails, and algae, duckweed, or pondweed, respectively. *Anopheles freeborni* was the only species present in 27% of

collections, was found with *An. earlei* and *An. punctipennis* in ~46% and 64%, respectively, and with both *An. earlei* and *An. punctipennis* in 15%, of the larval dipping collections conducted in the province.

Anopheles punctipennis was the most commonly collected species, with over 2000 specimens (44% of all larvae) obtained. They were mostly collected in the Niagara region (41% of *An. punctipennis*-positive collections), but were also common in the Manitoulin area (28%), British Columbia (15%), Ottawa (10%), and Windsor (6%). It was collected from the widest variety of habitats, including ditches, creeks, ponds, old quarries, swamps, marshes, lake and river edges, forest and ground pools, and artificial containers such as tires. *Anopheles punctipennis* was most commonly collected when both emergent and floating vegetation were present, but sometimes when one or both types were absent. A wide variety of vegetation types were present in *An. punctipennis*-positive collections, most often of which were algae, cattails, grasses, and duckweed, but many others were recorded as well. *Anopheles punctipennis* was collected both alone and with all other anopheline species examined, the only species included in this study to do so. It was collected most often with *An. quadrimaculatus s.l.*, in 50% of all *An. punctipennis*-positive larval dipping collections, but also with *An. earlei* (24%), *An. walkeri* (10%), *An. freeborni* (4%), and *An. barberi* (2%).

Anopheles quadrimaculatus s.l. was the second most abundant species collected, with over 1600 specimens comprising ~34% of all larvae obtained by larval dipping. Over half of all *An. quadrimaculatus s.l.* specimens were collected from the Niagara region (57%), followed by Ottawa (26%), Windsor (16%), and in very low numbers on Manitoulin Island (0.5%). In Niagara, most larvae were collected from ditches and creeks, ponds, and old quarries, but they were collected mainly from the edges of lakes and rivers in the Ottawa region and ditches, creeks, and marshes in the Windsor area. *Anopheles quadrimaculatus s.l.* larvae were found most often when both emergent and floating vegetation were present in the larval habitat, but sometimes when emergent vegetation only was present, and rarely when only floating vegetation was present or both types were absent. It was collected most often from larval habitats with grasses, cattails, algae, and duckweed present, but was also commonly collected with a variety of others as well.

Finally, *An. walkeri* was collected in very low numbers, with only 73 larvae collected during all five years of sampling, comprising only 1.5% of all specimens obtained, even though regions well within its known range were among those most intensely sampled. The majority of specimens were collected on Manitoulin Island, a first record for the area, with very few collected in the Windsor, Niagara and Ottawa regions. The abundance of adults collected from near large marshes such as those along the north shore of Lake Erie between Windsor and Niagara where very few larvae were collected by larval dipping suggests that this method is not an ideal collecting method for this species. *Anopheles walkeri* was collected most often from swamps and marshes, but were also present in ditches, creeks, and the edges of lakes and rivers. They were found most often when both emergent and floating vegetation were present, sometimes when only one type was present, and rarely when both were absent. The most common types of vegetation present in larval habitats of *An. walkeri* were cattails, grasses, algae, and duckweed. *Anopheles walkeri* was never the only species obtained in a larval dipping collection, and were found most often with *An. punctipennis* (80%), followed by *An. quadrimaculatus* (60%) and *An. earlei* (35%). This study represents the first detailed analysis of larval habitat data for the *Anopheles* species present in Canada.

Chapter Five:
Morphological, molecular, and ecological data: Results of an integrated approach to *Anopheles* systematics in Canada

5.1. Introduction

Classical *Anopheles* taxonomy is difficult because anophelines are known for both strong morphological interspecific similarities and pronounced morphological intraspecific variation. It is further complicated by the recognition that many *Anopheles*, once thought to be single species, are actually complexes of species that are difficult or impossible to identify morphologically, yet they differ in ecological or behavioural characteristics that affect their ability to effectively transmit pathogenic organisms. The "Anophelism without malaria" problem that challenged European scientists during the first half of the 20th century best illustrates the importance of being able to accurately identify vector species, since the disease was successfully controlled once vector species could be distinguished from their harmless relatives and mosquito abatement programs could be concentrated in the correct areas (Besansky 1999; Walton *et al* 1999b).

However, it is because of their potential to transmit diseases to human and other animals, combined with the absence of reliable morphological characters that can be used to discriminate among closely related species, that researchers have searched for alternative ways to identify *Anopheles* species accurately, particularly the members of species complexes. From the 1940s until the 1970s, these methods were based primarily on cytological data (i.e. polytene chromosome banding patterns), as well as genetic incompatibility data (i.e. offspring viability resulting from hybridization experiments). During the 1970s and 80s, biochemical methods of species identification were developed, including those involving cuticular hydrocarbons and enzyme variants. However, since the discovery of PCR-based techniques during the 1980s, such as random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RLFP), molecular methods have become the primary alternatives for species identification. In particular, since the development of automated DNA sequencing, the use of DNA sequences, such as the mitochondrial *cytochrome oxidase* I gene (COI) and the internal

transcribed spacer sequence 2 (ITS2), has become standard in studies of *Anopheles* systematics.

DNA sequences, particularly ITS2, are now commonly used to help elucidate and identify cryptic *Anopheles* species. Once distinct clusters of morphologically similar specimens are discovered based on molecular data, re-examination of morphological characters can sometimes reveal minor differences not previously recognized before the specimens were separated into their molecular groupings. Similarly, once the isomorphic members of the *An. gambiae* and *An. maculipennis* were identifiable based on polytene chromosome banding patterns, the ecological characteristics associated with each species, such as larval habitat and host preference, could then be determined and used to help identify vector species distributions more accurately. However, caution must be exercised, as there is no single method that can be used to reliably identify all anopheline species.

For example, while polytene chromosomes were instrumental in the discovery of anopheline species complexes, not all species have discernible differences in the banding patterns that can be used to identify them. Molecular data has led to the discovery of many cryptic species (Bickford *et al.* 2007), but they often remain taxonomically cryptic since traditional taxonomists are needed to name and describe the morphology of newly recognized molecular species (Schlick-Steiner *et al.* 2007). Phylogenetic studies using morphological and molecular data have demonstrated the importance of using multiple markers to generate more accurate hypotheses of species delineation and relationships among species. This is particularly important for *Anopheles* taxonomy, as closely related species may differ in some, but not all, possible detectable ways (i.e., minor changes in egg morphology revealed by SEM, distribution or larval habitat, allozyme profiles, DNA sequences, polytene chromosomes, etc.). Knowledge of the ecological characters associated with each species is of practical significance due to their role in the transmission of diseases to humans and animals.

The importance of a multidisciplinary approach to *Anopheles* systematics has long been recognized. Faran (1979) discussed the importance of an integrated approach and how the correlation of multiple data sets, including morphology, behaviour, ecology, vector potential, and cytogenetics, can not only help delimit existing species and

elucidate new ones, but sometimes can raise even more questions. Indeed, it seems as though the more is learned about *Anopheles* systematics, the more complicated the relationships among species seem to become. For example, *An. funestus* was thought to be two closely related species that were distinguishable based on a polytene chromosomal inversion, one of which was found significantly more often in indoor versus outdoor resting sites (Besansky 1999). Although, examination of the mitochondrial gene *cytb* and ITS2 sequences indicated that *An. funestus* was a single panmictic population, if separation of the species occurred very recently, this might not necessarily be evidence against reproduction isolation between the two species (Besansky 1999). Incongruence between different markers highlights the complexity that is often present among the closely related members of species complexes. There are many examples of cryptic species that differ from each other in only a few very minor ways (Bickford *et al.* 2007), thus illustrating how investigations of systematics benefit from the use of as any many datasets as possible.

Despite the presence of isomorphic species, morphology remains an important perspective from which to investigate questions of *Anopheles* systematics as specimens are traditionally identified morphologically prior to molecular analyses and, once cryptic species are discovered based on molecular data they can often be distinguished by minor morphological characters through morphometric analyses (Sáez and Lozano 2005; Schlick-Steiner *et al.* 2007). Therefore, morphology was chosen as the first perspective from which to study the anopheline specimens collected from across Canada. Since cryptic species are, by definition, difficult to identify based on morphology alone, methods other than morphology were also used. Due to many advantages of using DNA sequence data to both identify species and elucidate cryptic species (e.g., COI and ITS2), *Anopheles* mosquitoes from Canada were also examined using molecular data. Finally, due to a lack of bionomic data known for many of the species whose ranges extend into Canada (especially within the northern regions of their ranges), and it's importance for the success of potential larval mosquito control programs, ecological data associated with the larval habitats of *Anopheles* species in Canada were also analysed. Combining the results of these types of data in an integrated approach to *Anopheles* systematics, incongruence between morphological and molecular data was revealed and the presence

of two cryptic taxa was discovered. The following is a brief summary of the results from each type of analysis (morphological, molecular, and ecological), followed by a discussion of the importance of using an integrated approach and the overall conclusions of the study.

5.2. Morphology

Adult and larval mosquitoes were identified using the morphology-based key to the North American mosquito species (Darsie and Ward 2005) to ensure that any species currently known only from the USA, whose ranges may have extended north into Canada in recent years, were not missed during identification of the specimens obtained during this study. This key includes many species not known from Canada, including two species, *An. perplexens* and *An. smaragdinus*, which are morphologically very similar to native species, *An. punctipennis* and *An. quadrimaculatus s.s.*, respectively. Many specimens collected during this study keyed out to these cryptic species, and since their distributions appear to approach the Canada-USA border, alternative methods were sought that would allow confirmation of the presence of these cryptic species in Canada, including ITS2 sequence data (discussed below) and scanning electron microscopy of eggs, both of which are known to be able to reliably identify both *An. perplexens* and *An. smaragdinus* from their respective siblings.

Examination of the eggs obtained from adult females collected in the Niagara region using the SEM available at Brock University resulted in good quality images for 19 *An. punctipennis* egg batches (SCP ratio >0.5), six females with intermediate SCP ratios (i.e. >0.33 and <0.5), and one *An. perplexens* female (SCP ratio <0.33). However, all 26 batches of eggs were identified as *An. punctipennis* based on the morphological differences described by Linley and Kaiser (1994). Since only one egg batch was obtained from an *An. perplexens*-type female, the results of the SEM data regarding the presence of this species in Canada were inconclusive.

The eggs of *An. quadrimaculatus s.l.* females were also examined using scanning electron microscopy for morphological evidence that *An. smaragdinus* is present in Canada. However, few egg batches were obtained and all were from females identified

as *An. quadrimaculatus s.s.*, and the angle of the eggs in many images did not allow the morphological character used to distinguish these two species to be observed. Thus, the results of the SEM data were, again, inconclusive. While SEM examination of egg morphology is a reliable means of identification for many closely related cryptic species, obtaining a sufficient number of samples for analysis was difficult for a number of reasons. After adult females were collected by CDC light traps or landing aspirations, some died before their return to the lab. Some females refused to take blood meals from the hosts provided or died during the process, and other did not lay any eggs. Therefore, although potentially reliable, SEM examination of eggs as a method for identifying cryptic species can be difficult unless collection locations are previously known because obtaining IPBs from field-collected females is a labour-intensive process.

The next species examined morphologically for evidence of cryptic species was *An. earlei*, which has the broadest geographic range in North America of all species examined in this study, from near the tree line in northern Canada to the midwestern United States in the south (Figure 1.9). Since *An. earlei* occurs across a very broad range and in a wide variety of different ecosystems, the potential presence of cryptic species within this taxon was considered (Kitzmiller 1959), and morphological variation in a larval trait was observed (i.e., presence of additional ATPs), this character was examined in all *An. earlei* larvae obtained. The presence of additional accessory tergal plates (ATPs) is a diagnostic character for *An. freeborni* in both keys available for the identification of mosquitoes (Darsie and Ward 2005; Wood *et al.* 1979), but is not mentioned or present in descriptions or illustrations of *An. earlei* larvae in any other relevant publications (Belton 1983; Carpenter and LaCasse 1955). Therefore, larvae collected from various regions across Canada were examined for regional differences in the number of additional ATPs that might indicate the presence of a cryptic species within *An. earlei*.

Additional ATPs were absent from all Newfoundland larvae, and were present in all larvae from Niagara, but samples sizes were very low for both regions (three and four, respectively). Larvae from all other regions included in the analysis (i.e., Algonquin, Manitoulin Island, Ottawa, and British Columbia) exhibited high levels of variation in the number of additional ATPs present. Additional ATPs were present in 65% of all larvae

examined, and of those, the number of spots was highly variable, with anywhere from one to 10 extra ATPs per larva. The high number of individuals with an intermediate number of ATPs (one to seven), combined with no detectable pattern in their presence based on geographic distribution, suggests that this particular trait is highly polymorphic in *An. earlei* but does not necessarily indicate the presence of a cryptic species within this taxon.

5.3. Molecular Data

Due to the many advantages of DNA sequence data as a method for the identification of species and elucidation of cryptic species over more traditional ones (i.e. morphological and cytological methods), *Anopheles* mosquitoes from across Canada were analysed using three molecular markers, namely COI, ITS2, and ITS1. The use of the 650bp "barcoding" region of the COI gene is now well established in the literature as a species identification tool that works well for almost all species examined to date. However, because this particular region of the COI gene has already been studied in Canadian anophelines (Cywinska *et al.* 2008), and the 800bp region located at the 5' end of the gene has been shown to be more informative with respect to the elucidation of cryptic species in a related family of flies, the black flies (Cywinska *et al.* 2010), I chose to examine the 5' 800bp section of COI gene. Since a review on the use of the internal transcribed spacer sequences of the ribosomal DNA (rDNA) to identify mosquitoes of the genus *Anopheles* was published (Collins and Paskewitz 1996), ITS2 sequence data have become standard in investigations of anopheline systematics and have uncovered the presence of cryptic species in many taxa. ITS2 was therefore selected as the second molecular marker with which to examine the *Anopheles* mosquitoes from Canada. The other rDNA internal transcribed spacer sequence, ITS1, has only been examined in a few anopheline species, with a higher level of intraspecific variation observed compared to ITS2. Therefore, ITS1 was selected as the third molecular marker.

Analysis of the COI data revealed distinct intraspecific clusters of specimens within two of the species examined. *An. punctipennis* and *An. perplexens* samples resulted in two groups with a sequence divergence greater than 2%, higher than the 0.5%

average intraspecific sequence divergences found in mosquitoes using the barcoding region of COI (Cywinska *et al.* 2008). The groups differed by region of collection, with *An. punctipennis* specimens from BC in one group and all *An. punctipennis* and *An. perplexens* specimens from all four regions in Ontario (WE, NI, OT, and NO) in another, and not by morphological identification as expected. *An. walkeri* sequences also formed two distinct groups: one including specimens from Manitoulin Island and the other with specimens collected at Long Point Provincial Park, with sequence divergence between the two groups greater than 7%, much higher than expected for a single species.

The four remaining species included in the study all had average sequence divergence levels of 0.6% or less. *An. barberi* and *An. freeborni* specimens were collected from relatively smaller geographic regions (NI and BC, respectively) and had the lowest levels of intraspecific variation, 0.0% and 0.1%, respectively. For a species with such a large distribution, average sequence divergence was very low among *An. earlei* specimens, only 0.2%, with a maximum divergence of 0.6%. Finally, sequence divergence levels of specimens identified as *An. quadrimaculatus s.s.* and *An. smaragdinus* were also within the normal intraspecific range, with an average of 0.6% and a maximum of 1.2%, and no molecular groups were formed that corresponded to groupings based upon morphological identification of specimens.

Analysis of the ITS2 data revealed similar patterns of intraspecific variation as seen in the COI sequences. The size of the ITS2 fragments ranged from 379 nucleotides (*An. barberi*) to approximately 800bp in *An. earlei*, which were species-specific and usually identical for all specimens within a species (Figure 3.10). Because a large section of the *An. earlei* sequence could not be sequenced, the 800bp fragment was trimmed to a 318bp sequence for analysis. Two species had zero nucleotides (nts) differences among all specimens examined; *An. freeborni* (n=40, 0/422 nt changes) and *An. quadrimaculatus s.l.* (n=73, 0/395 nt changes), including those identified morphologically as *An. smaragdinus* (n=8). Despite a small sample size and all specimens being from the same region (NO), *An. barberi* had a single nucleotide change in one specimen (n=18, 1/275 nt changes). The ITS2 sequences of *An. earlei* from BC (n=5) differed from specimens from all other regions (n=27), including RQ and NF, by 2/318 nucleotides. This could

indicate that populations east and west of the Rockies have become reproductively isolated very recently and they cannot yet be differentiated using COI.

The two remaining species included in the study revealed sufficient levels of intraspecific variation in the ITS2 region to suggest the presence of cryptic species. ITS2 sequences of specimens identified morphologically as *An. punctipennis* and *An. perplexens* produced two distinct groups that differed by 27/375 nucleotides. The first group included all specimens from BC, which were identified as *An. punctipennis* based on morphology (n=11), and the second included all other specimens, which were from all four regions in Ontario and identified morphologically as *An. punctipennis* and *An. perplexens* (n=75). These results provide further evidence for the presence of cryptic species within *An. punctipennis* that differ based on geographic origin, i.e., east and west of the Rockies. The ITS2 sequences of specimens identified morphologically as *An. walkeri* also formed two distinct groups that differed by 73/231 nucleotides, the first including specimens from Manitoulin Island (n=6) and the second with specimens from Long Point Provincial Park (n=6). As in COI, *An. walkeri* ITS2 revealed the highest level of intraspecific variation, with the same difference between groups being collection location (NO vs. LP). Therefore, both COI and ITS2 data were successful in accurately identifying six of the seven anopheline species known from Canada based on morphological data, and provided evidence for the presence of two additional species in Canada.

The results of the ITS1 data, however, were not as straightforward. Since published primers for the ITS1 region were based on African or Australian sequences, novel primers were required to amplify ITS1 in North American species. The primers designed for this study amplified the ITS1 region in five of the six species examined, all except for *An. barberi*, the only species belonging to the Plumbeus Group (Figure 1.6). The size of the ITS1 fragments of the other five species ranged from ~600-4500bp in length (Figure 3.15). *An. walkeri* and *An. quadrimaculatus* produced single bands at ~600bp and 800bp, respectively, which were easily sequenced in both directions and resulted in reliable consensus sequences. *An. walkeri* ITS1 sequences resulted in two distinct groups, the first including specimens from Manitoulin Island (n=9; 483bp) and the second with specimens from Long Point Provincial Park (n=10; 490bp), with 39/490

variable sites or a sequence divergence level of 8%, consistent with the COI and ITS2 results for this species.

The ITS1 sequence results for *An. quadrimaculatus s.l.* specimens, however, were not consistent with COI and ITS2 results for this species, i.e., low sequence divergence levels in both markers suggested that cryptic species were not present in this taxa. However, *An. quadrimaculatus s.l.* ITS1 sequences resulted in two distinct groups, the first including specimens from NI, WE, and OT (n=35; 729bp), and the second also with specimens from NI, and WE (n=10; 756bp). In addition to the large difference in size (27bp), there were 335/756 variables sites, or a sequence divergence of 56%, between the two groups. Whereas the majority of variable sites between the two *An. walkeri* groups were concentrated in the middle of the sequences (Figure 3.20), the variable sites between the two groups of ITS1 sequences between *An. quadrimaculatus s.l.* groups were spread evenly throughout the sequence (Figure 3.19).

Anopheles earlei and *An. freeborni* also produced single ITS1 bands, but both were much larger at ~4000 and 4500bp, respectively, and the sequencing and determination of consensus sequences was much more difficult. Sequencing in the first ~800bp in each direction was attempted for both species, but was only successful in *An. freeborni*, resulting in good quality sequences for the first ~800bp and last ~750bp of the ITS1 region. Combined there were fewer than 10 variable sites within the entire ~1550bp of *An. freeborni* ITS1 combined sequence, or a low sequence divergence of 0.7% which is consistent with the COI and ITS2 findings for this species.

In *An. earlei*, the sequencing reaction begins to break down after the first ~350bp in the forward direction, becoming completely unreadable after ~650bp. Sequencing in the reverse direction was even less successful, with sequences from three specimens excluded due to poor quality overall. After establishing common start and stop points for the remaining reverse sequences, they were ~750bp in length, and identical for the first 500bp, but then the signal rapidly deteriorates and the sequence becomes unreliable. This suggests intraindividual variation in the ITS1 region of *An. earlei*, similar to that seen in *An. gambiae* and *An. arabiensis* (Collins *et al.* 1999), the *An. punctulatus* group (Bower *et al.* 2009) and *An. farauti s.s.* (Bower *et al.* 2008). Therefore, it will likely be necessary to use closing techniques to obtain individual sequence variants combined with the design

of step-wise primers to sequence the entire ~4000bp sequences; then this molecular marker may be useful in studies of population genetics.

Finally, *An. punctipennis*, the last species for which ITS1 was successfully amplified, resulted in multiple bands that varied in size from ~800bp to 3000bp, with most specimens having two to three bands, the most common of which were ~2500bp, 3000bp, and 1250bp in length (Figure 3.17). Band intensity also varied, suggesting that some ITS1 variants were more common in that individual than others. However, because most specimens resulted in multiple bands, sequencing of the *An. punctipennis* ITS1 sequences was not possible without further treatment (e.g. band extraction and purification). The only specimens to result in single ITS1 bands were those of four *An. punctipennis* individuals from British Columbia (Figure 3.18), which were ~2000bp in length and resulted in ~750bp of readable ITS1 sequence in both the forward and reverse directions, which were almost identical throughout. This difference in number and size of the ITS1 fragments between BC and ON *An. punctipennis* is consistent with the COI and ITS2 results and provides further evidence for the presence of eastern and western sibling species in this taxon.

5.4. Ecology

In addition to morphological and molecular data, ecological data were also analysed for all anopheline species collected for this study. Larval habitat characteristics (including date of collection, GPS coordinates, water body types, emergent/floating vegetation types, etc.) were recorded for 350 unique larval dipping collections, 221 of which contained *Anopheles* larvae. However, some areas or habitat types were sampled more intensely than others, which complicated the analysis of the ecological data collected. For example, the first two field seasons involved random sampling in 4 main regions of Ontario (WE, NI, OT, and NO) plus a long-distance collecting trip to British Columbia in 2005 and one to Newfoundland in 2006, but the 2007-2009 field seasons focused on locating sources of potential cryptic species, *An. perplexens* and *An. smaragdinus*, in the Niagara region, which resulted in the majority of specimens being collected from this region (Figure 4.1). Also, to increase the number of collections made

in each region, accessibility of the larval habitat was taken into consideration and many collections were made in water body types that were easily reached by roads and trails, such as ditches (Table 4.8). Therefore, the results of the analyses of regional abundance, larval habitat, and species associations were a reflection of the particular habitats located during the course of this study, and not necessarily that of all possible larval habitat associations for each species that occurs in Canada.

The larval habitat of *An. barberi* was the most distinct of all anophelines included in the study, found mainly in tires that were placed in a Niagara woodlot to study competition between other tree hole-breeding mosquito species. Recorded primarily from tree holes in the literature, very few larvae of *An. barberi* were obtained from this habitat type, due to the difficulty in reaching them without special equipment. Therefore, overall, very few *An. barberi* specimens were collected through larval dipping. Larvae were never associated with any type of floating or emergent vegetation due to their restriction to tree holes and tires and the only species ever found associated with *An. barberi* was *An. punctipennis* (including those identified as *An. perplexens*), as it too was found in tires (but not tree holes). However, this species likely occurs throughout southern Ontario, wherever suitable habitat (hardwood forest) exists.

Even fewer *An. freeborni* specimens were collected during the study, due to the combination of limited larval dipping collections made in British Columbia (the only region in Canada where it occurs) than other regions, and the difficulty in finding suitable *Anopheles* habitats during that collecting trip. Almost all *An. freeborni* specimens were collected from ponds on farms and golf courses, but some from ditches as well. *An. freeborni* was always associated with one or more types of emergent and floating vegetation, the most common of which were grasses, algae, and cattails. It was usually found with *An. punctipennis* (64% of *An. freeborni*-positive collections), *An. earlei* (46%), or both species (15%), but could also be the only species obtained from a larval dipping collection (27%).

The species for which the least number of larvae was collected was *An. walkeri*, which was not expected since the most intensely sampled regions (WE, NI, and OT) were well within this species' known range and *An. walkeri* was said to be the most common anopheline in southern Ontario in the summer months (Wood *et al.* 1979). The majority

of specimens was collected from Manitoulin Island, for which there were no previous records but is very near the known northern limit of its range (Figure 1.13). *Anopheles walkeri* was collected most often from marshes, swamps, and ditches and usually when both emergent and floating vegetation were present in the larval habitat. The most common types of vegetation associated with *An. walkeri* larvae were cattails, grasses, and algae. This species was never the only one obtained in a larval dipping collection, and was found most often with *An. punctipennis* (80% of *An. walkeri*-positive collections), *An. quadrimaculatus* (60%), and *An. earlei* (35%), with at least two of the three other marsh-inhabiting species in 70% of all *An. walkeri*-positive collections.

The two remaining species, *An. punctipennis* and *An. quadrimaculatus s.s.*, were both found in very large numbers, and from a wide variety of habitat types. Most *An. quadrimaculatus s.l.* specimens were collected in NI, followed by OT and WE, but a few specimens were collected from NO as well. Larvae of *An. quadrimaculatus s.l.* were found most often in ditches and creeks, ponds, and from the edges of lakes and rivers, but was collected from a wide variety of other habitats as well (Figure 4.9). *Anopheles quadrimaculatus s.l.* larvae were usually associated with both emergent and floating vegetation, and less often when only emergent vegetation was present. The most common types of associated vegetation were algae, grasses, and cattails, but many more types were also found (Figures 4.13 and 4.14). *Anopheles quadrimaculatus s.l.* was collected most often with *An. punctipennis* (in 78% of all *An. quadrimaculatus*-positive larval dipping collections), but also with *An. earlei* (17%), and *An. walkeri* (11%).

Anopheles punctipennis was the most common species found in all of the five main regions analysed, comprising ~30-70% of all specimens collected in each region. *An. punctipennis* larvae were collected from the widest variety of habitats, including eight out of ten possible types (Figure 4.5). A similar pattern was observed for specimens identified morphologically as *An. perplexens* (Figure 4.6). While alvars or alvar-type habitats (i.e. exposed limestone) were present in each of the three regions where *An. perplexens*-type specimens were collected (NI, OT, and NO) (Figure 4.8), there were no sites in which *An. perplexens*-type specimens were the only species collected. The large number of *An. perplexens*-type specimens collected from limestone habitats in NI was definitely due in part to increased effort to locate such habitats in this area once the first

few were discovered. However, a number of natural and man-made alvars, plus quarries both old and active, were found in the Niagara region (not shown in Figure 4.8). The large number of *An. perplexens*-type individuals combined with the location of alvars in regions where specimens were collected suggested that this cryptic species might be present in this region, not far from where other populations have apparently been found (Figure 1.15).

Specimens identified as *Anopheles punctipennis* and *An. perplexens* based on morphological data were found most often when floating and emergent vegetation was present, but also when one, the other, or both were absent. The most common types of emergent and floating vegetation were present in larval habitats of *An. punctipennis*- and *An. perplexens*-type specimens were algae, cattails, and grasses, but were also found with a wide variety of other types. It was the only species collected with all other species examined in the study. While *An. punctipennis* was sometimes the only species found (21% of *An. punctipennis*-positive collections), it was found with *An. quadrimaculatus s.l.* (50%), *An. earlei* (24%), *An. walkeri* (10%), *An. freeborni* (4%), and *An. barberi* (2%). Therefore, *An. punctipennis* appears to be the most ecologically diverse species present in Canada.

5.5. Importance of an Integrated Approach

The advantage of having more than one perspective from which to examine the specific status of morphologically similar taxa is apparent when one considers the importance of the ability to distinguish among closely related species that differ in their potential to spread diseases like malaria, as is the case for *Anopheles* mosquitoes. Many techniques are now available to examine closely related anopheline species, from polytene chromosomes and hybridization experiments, to PCR-based methods such as RAPD and direct sequencing of taxonomically informative DNA sequences. Interconnected to all of these facets of differentiation among species are the ecological differences (as well as physiological and behavioural ones) associated with each species. Ecological characters were once often the first insight into the presence of cryptic species (e.g. fresh water versus salt water species in the *An. gambiae* complex), and can often be

associated with cryptic taxa once they are identifiable using other methods, such as DNA barcoding (e.g., the identification of different host plants for multiple isomorphic host-specific butterfly species once thought to be a single, generalist species).

The advantage of multiple types of data was evident in this examination of *Anopheles* species from Canada given that examples of incongruence between the types of data analysed were found. One of the first unexpected results of this study was that COI and ITS2 data did not provide molecular evidence for the presence of *An. perplexens* in Canada, whereas morphological and ecological data seemed to suggest that it was present. Although the adult and larval characters used to distinguish these species were known to be unreliable, adults collected from southern Ontario that were identified morphologically as *An. perplexens* fit the species description provided in Bellamy (1956), including their smaller and darker appearance in general compared to those of *An. punctipennis*. Data that led to the suspicion that *An. perplexens* was present in Canada included the high numbers of *An. perplexens*-type specimens identified from field collections, the fact that they were collected not far from supposed known locations in the USA for this species, and that the regions where most *An. perplexens*-type specimens were collected also contained potential suitable habitat (i.e., limestone) for this species. However, both COI and ITS2 results revealed very low levels of intraspecific sequence divergence for all *An. punctipennis*- and *An. perplexens*-type specimens from Ontario, indicating the presence of a single panmictic species. Although very unlikely, it is possible that this represents a case where separation between species occurred so recently that differences have not yet developed in these particular markers. However, since siblings reared from the same isofemale progeny broods resulted in a wide range in wing spot sizes (Figure 2.25), and "*An. perplexens*" was never the only species identified in a larval dipping collection, this suggests that specimens identified as *An. perplexens* are instead morphological variants of *An. punctipennis*.

At the same time, COI and ITS2 results of *An. punctipennis* uncovered the presence of an unsuspected cryptic species. Both markers revealed two distinct groups of specimens with significant levels of intraspecific variation between them; the *An. punctipennis* specimens from BC formed the first group, and all remaining specimens (*An. punctipennis* and *An. perplexens* from all regions in Ontario) in the second.

However, in a phylogenetic study of the Nearctic *Anopheles maculipennis* species group using the D2 expansion region of the 28S rRNA, Porter and Collins (1996) found that *An. punctipennis* samples formed two distinct groups, one with California specimens (*An. punctipennis* W) and one with specimens from Wisconsin and Illinois (*An. punctipennis* E), with significant variation between the two groups. Further evidence for the presence of a pair of sibling species in this taxon comes from the absence of *An. perplexens*-type specimens collected from British Columbia despite a large number of *An. punctipennis* specimens collected from this region, as well as the lack of multiple ITS1 bands produced by the *An. punctipennis* specimens from BC. In addition, while the distribution range of *An. punctipennis* extends from coast to coast in North America, it narrows drastically at the Rocky Mountains, somewhat separating the western and eastern populations (except for a small region in the midwestern states of Montana, Idaho, and Wyoming), likely restricting gene flow between them.

A multidisciplinary approach was also beneficial in clarifying the specific status of *An. earlei* from geographically distant locations across Canada, including British Columbia, northern Quebec, Newfoundland, and almost all regions in Ontario. Like many other species with broad (e.g., continental) ranges, such as *An. maculipennis* (Europe) and *An. gambiae* (Africa), *An. earlei* is known from a wide variety of habitats throughout its range. However, due to a lack of importance with respect to disease transmission, detailed investigations of the systematics of *An. earlei* are lacking, and it has never been analysed using molecular data from a wide variety of locations. Morphological analysis of *An. earlei* larvae revealed the presence of a trait diagnostic for *An. freeborni* larvae in all available identification keys, which was found to be highly variable in number and placement along the dorsal surface of the abdomen (Figure 2.20). This polymorphism was thought to suggest the potential for cryptic species in this taxon, but if cryptic species were present in *An. earlei*, they would also be based on collection location (BC vs. ON, RQ, and NF) and not morphological identifications. COI sequence divergence levels were lower than expected for two distinct species, but there was weak support for one group that included all four *An. earlei* specimens from BC specimens and three specimens from AL (Figure 3.4). A similar separation of two groups was also seen in the 318bp *An. earlei* ITS2 sequences, with 2 nucleotides shared among the BC

specimens that differed from those collected from all other regions. Like *An. punctipennis*, *An. earlei* is present from coast to coast (Figure 1.9), however, its range is not constricted in the middle like *An. punctipennis* (Figure 1.11), and thus was not subject to as much reduction in gene flow, explaining the lower levels of sequence divergence seen in *An. earlei*.

Finally, the use of molecular data revealed the presence of an unexpected putative cryptic species within *An. walkeri*. All three molecular markers examined in this study resulted in the formation of the same two molecular groupings that corresponded to the regions in which the specimens were collected, i.e., those from Manitoulin Island and those from Long Point Provincial Park. The distribution of *An. walkeri* follows a strange pattern and appears to approach the island from the east and the west (Figure 1.13), although it is not clear if the absence of records in northwestern Ontario is due the absence of *An. walkeri* in this region, or to a lack of sampling. However, the levels of intraspecific variation in COI, ITS2, and ITS1 sequences appear sufficiently high to warrant the confirmation of the presence of a new cryptic species within *An. walkeri*.

The results of these morphological, molecular, and ecological analyses illustrate the importance of using an integrated approach to investigate systematic questions in *Anopheles*. The advantages of using molecular data as a species diagnostic tool are apparent given the discovery of two cryptic species with the six anophelines examined in this study, and the recognition that the morphological variations observed in *An. punctipennis* and *An. quadrimaculatus s.s* that appeared to suggest the presence of cryptic species *An. perplexens* and *An. smaragdinus*, respectively, were not indicative of cryptic taxa. Therefore, the increased variation in morphological traits observed in these species should be considered if using the key to North American mosquito species (Darsie and Ward 2005). As morphology can sometimes be influenced by environmental conditions in *Anopheles* (e.g., Peters 1943; Service 1964), the possibility that water chemistry in the limestone larval habitats could be responsible for the minor morphological differences observed between specimens identified as *An. punctipennis* and *An. perplexens* was considered. However, hybridization studies by Kreutzer and Kitzmiller (1971b) revealed high levels of mortality and infertility in the offspring of crosses (by artificial mating techniques) between *An. punctipennis* and *An. perplexens*, and similar studies of their

polytene chromosomes revealed regions of synapsis and asynapsis, where chromosomal inversions between the two species were present (Kreutzer and Kitzmiller 1971a). This example highlights the importance of obtaining as many data sets as possible to clarify questions of *Anopheles* systematics.

5.6. Conclusions

Prior to the current study, seven species of *Anopheles* mosquitoes were known from Canada, including *An. barberi* (tree hole specialist in southern Ontario and Quebec), *An crucians s.l.* (only in southwestern Ontario), *An. earlei* (present throughout Canada), *An. freeborni* (only in southern British Columbia), *An. punctipennis* (present in parts of southern Canada), *An. quadrimaculatus s.l.* (only in southern Ontario and Quebec), and *An. walkeri* (Saskatchewan eastward to Nova Scotia). Despite effort to collect all seven species, *An. crucians s.l.* specimens were not collected during the course of study and, therefore, could not be included in the morphological, molecular, or ecological analyses conducted.

Morphological identification of specimens collected during the 2005-2009 field seasons using standard keys suggested that two cryptic species might be present in Canada, including *An. perplexens* (morphologically similar to *An. punctipennis*) and *An. smaragdinus* (a cryptic member of the *An. quadrimaculatus* species complex). In addition, a larval character used to distinguish *An. freeborni* from the remaining anophelines was observed in *An. earlei*, in which it was highly variable in number and placement on abdominal tergites. Therefore, morphological examination of specimens revealed the potential presence of cryptic anopheline species in Canada and the degree of variation in these polymorphic traits was quantified.

Molecular data also revealed the potential presence of two cryptic species, however, molecular species corresponded to collection location, and not to morphological identifications as expected. Examination of molecular markers commonly used to identify species and elucidate cryptic members of species complexes (i.e., COI and ITS2) resulted in the formation of two distinct molecular groups within two of the six species examined. *Anopheles punctipennis* specimens formed two groups, the first containing specimens collected from British Columbia and the second comprised of specimens

collected throughout Ontario, which exhibited sequence divergence levels higher than that expected for a single panmictic species. Even more distinct with higher levels of sequence divergence were two groups within *An. walkeri*, the first of which included specimens from Manitoulin Island, Ontario and the second of which comprised those from Long Point Provincial Park. However, specimens identified morphologically as *An. perplexens* and *An. smaragdinus* did not differ significantly from native species *An. punctipennis* and *An. quadrimaculatus s.l.*, respectively. Therefore, molecular data provided evidence for the presence of putative cryptic species, i.e., *An. punctipennis* BC (western species) and *An. punctipennis* ON (eastern species), and *An. walkeri* NO (northern species) and *An. walkeri* LP (southern species).

Although ecological data were not analysed with respect to the discovery of cryptic species *per se*, the ecological data associated with larval dipping collections were examined to determine if the larval habitats of potential cryptic species differed from those of native *Anopheles* species in Canada, and to provide current larval habitat descriptions for each species examined. In British Columbia, *Anopheles* species were most commonly collected from ponds, and, in Ontario, they were collected most often from ditches and creeks, despite the common nickname of "marsh" species for most anophelines present in Canada.

This study highlights the difficulty imposed by morphological identification of specimens, as intraspecific morphological variation was quite pronounced within some species (e.g., *An. punctipennis* with both large and greatly reduced pale-scaled wing spots) but interspecific morphological variation between other species not apparent (e.g., *An. walkeri* "northern species" and *An. walkeri* "southern species"). The advantage of an integrated approach using multiple types of data was clear in this study, as morphological data suggested the presence of certain cryptic species, but molecular data did not confirm their presence in Canada, providing evidence for other cryptic species instead.

Bibliography

Aitken, T.H.G. 1945. Studies on the anophline complex of western North America. Eds. E.O. Essig, S.B. Freeborn, H.S. Smith. Volume 7, No. 11, pp. 273-364. University of California Press. Berkeley and Los Angeles, USA.

Baker, R. H. and J. B. Kitzmiller. 1964. Salivary gland chromosomes of *Anopheles punctipennis*. Journal of Heredity. 55: 9-17.

Ball, S.L., P.D.N. Hebert, S.K. Burian and J.M. Webb. 2005. Biological identification of mayflies (Ephemeroptera) using DNA barcodes. *Journal of North American Benthological Society*. 24(3), 508-524.

Barr, A.R. 1988. The *Anopheles maculipennis* complex (Diptera: Culicidae) in western North America. Biosystematics of Haematophagous Insects (ed. M.W. Service). Systematics Association. Special Volume No. 37, pp. 19-24. Clarendon Press, Oxford.

Barret, R.D.H. and P.D.N. Hebert. 2005. Identifying spiders through DNA barcodes. *Canadian Journal of Zoology*. 83: 481-491.

Beach, R.F., D. Mills, and F.H. Collins 1989. Structure of ribosomal DNA in *An. albimanus* (Diptera: Culicidae). *Annals of the Entomological Society of America*. 82(5): 641-648.

Beckingham, K. 1982. Insect rDNA. *The Cell Nucleus* (Busch, H. and L. Rothblum, eds). Academic Press, New York. 205-269pp.

Behura, S.K. 2006. Molecular marker systems in insects: current trends and future avenues. *Molecular Ecology*. 15: 3087-3113.

Bellamy, R.E. 1956. An investigation of the taxonomic status of *Anopheles perplexens* Ludlow 1907. *Annals of the Entomological Society of America*. 49: 515-529.

Belton, P. 1983. The Mosquitoes of British Columbia. British Columbia Provincial Museum. Handbook 41. 189pp.

Berlocher, S.H. 1984. Insect Molecular Systematics. *Annual Review of Entomology*. 29: 403-433.

Berrang-Ford, L., J.D. MacLean, T.W. Gyorkos, J.D. Ford, and N.H. Ogden. 2009. Climate change and malaria in Canada: A systems approach. *Interdisciplinary Perspectives on Infectious Diseases*. Volume 2009, Article ID 385487, 13 pp. doi:10.1155/2009/385487.

Besansky, N.J. 1999. Complexities in the analysis of cryptic taxa within the genus *Anopheles. Parassitologia.* 41: 97-100.

Bickford, D., D.J. Lohman, N.S. Sodhi, P.K.L. Ng, R. Meier, K. Winker, K.K. Ingram, and I. Das. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution.* 22(3): 148.155.

Bower, J.E., R.D. Cooper, and N.J. Beebe. 2009. Internal repetition and intraindividual variation in the rDNA ITS1 of the *Anopheles punctulatus* group (Diptera: Culicidae): multiple units and rates of turnover. *Journal of Molecular Evolution.* 68: 66-79.

Bower, J.E., M. Dowton, R.D. Cooper, and N.J. Beebe. 2008. Intraspecific concerted evolution of the rDNA ITS1 in *Anopheles farauti* senso stricto (Diptera: Culicidae) reveals recent patterns of population structure. *Journal of Molecular Evolution.* 67: 397-411.

Brooker, R.J., E.P. Widmaier, L.E. Graham, P.D Stiling, C. Hasenkampf, F.F. Hunter, M.J. Bidochka, and C.D. Riggs (Eds.). 2010. Biology, Canadian Edition. McGraw-Hill Ryerson. USA. 1317 pp.

Brown, B. V. 1993. A further chemical alternative to critical- point-drying for preparing small (or large) flies. Fly Times. 11: 10

Calisher, C. H. 1994. Medically important arboviruses of the United States and Canada. *Clinical Microbiology Reviews.* 7: 89-116.

Carpenter, S.J. and W.J. LaCasse. 1955. Mosquitoes of North America (North of Mexico) University of California Press. Los Angeles.

Caterino, M.S., S. Cho and F.A.H. Sperling. 2000. The Current State of Insect Molecular Systematics: A thriving Tower of Babel. *Annual Review of Entomology.* 45: 1-54.

Chen, B., R.K. Butlin, and R.E. Harbach. 2003. Molecular phylogenetics of the Oriental members of the Myzomyia Series of the *Anopheles* subgenus *Cellia* (Diptera: Culicidae) inferred from nuclear and mitochondrial DNA sequences. *Systematic Entomology.* 28(1): 57-69.

Clements, A.N. 1992. The biology of mosquitoes. Vol 1. Development, nutrition, and reproduction. CABI, New York. 509 pp.

Collins, F.H. and S.M. Paskewitz. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Molecular Biology.* 5(1): 1-9.

Collins, F.H., S.M. Paskewitz, and V. Finnerty. 1989. Ribosomal RNA genes in the *An. gambiae* species complex. Advances in Disease Vector Research Volume 6 (K.F. Harris, ed). 1-28pp. Springer, New York.

Collins, F.H., C.H. Porter, and S.E. Cope. 1990. Comparison of rDNA and mDNA in the sibling species *Anopheles freeborni* and *hermsi*. *American Journal of Tropical Medicine and Hygiene*. 42(5): 417-423.

Coluzzi, M., A. Sabatini, V. Petrarca, A.P. Bianchi-Bullini, and L. Bullini. 1977. Genetic differentiation in the species of the *Anopheles gambiae* complex. *Atti dell'Associazione Genetica Italiana*. 22: 147-149.

Coluzzi, M., A. Sabatini, V. Petrarca, and M.A. Di Deco. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 73(5): 483-497.

Coluzzi, M., A. Sabitini, A. della Torre, M.A. Di Deco, and V. Petrarca. 2002. A polytene chromosome analysis of the *Anopheles gambiae* species complex. *Science*. 298: 1415-1418.

Constantini, C., D. Ayala, W.M. Guelbeogo, M. Pombi, C.Y. Some, I.H.N. Bassole, K. Ose, J.-M. Fotsing, N'F. Sagnon, D. Fontenille, N.J. Besansky, and F. Simard. 2009. Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in *Anopheles gambiae*. *BMC Ecology*. 9: 16, 27 pp. doi: 10.1186/1472-6785-9-16

Copeland, R.S. and G.B. Craig Jr. 1990. Habitat segregation among treehole mosquitoes (Diptera: Culicidae) in the Great Lakes region of the United States. *Annals of the Entomological Society of America*. 83(6): 1063–1073.

Cornel, A.J., C.H. Porter, and F.H. Collins. 1996. Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. *Journal of Medical Entomology*. 33(1): 109-116.

Cywinska, A., M.A. Hannan, P.G. Kevan, R. Roughley, M. Iranpiur and F. F. Hunter. 2010. Evaluation of DNA barcoding and identification of new haplomorphs in Canadian deerflies and horseflies *Medical and Veterinary Entomology*. 24(4): 382-410.

Cywinska, A., F.F. Hunter, and P.D.N. Hebert. 2008. Identifying Canadian mosquito species through DNA barcodes. *Medical and Veterinary Entomology*. 20: 413-424.

Darsie Jr., R.F. and R.A. Ward. 2005. Identification and geographic distribution of the mosquitoes in North America, north of Mexico. University Press of Florida, USA. 383pp.

Dawson, M.N. and D.K. Jacobs. 2001. Molecular evidence for cryptic species of *Aurelita aurita* (Cnidaria, Scyphozoa). *The Biological Bulletin*. 200: 92-96.

Debboun, M. and R.D. Hall. 1992. Mosquitoes sampled from treeholes and proximate artificial containers in Central Missouri. *Journal of Entomological Science.* 27(1): 19-28.

Drebot, M.A., R. Lindsay, I.K. Barker, P.A. Buck, M. Fearon, F. Hunter, P. Sockett, and Harvey Artsob. 2003. West Nile virus surveillance and diagnostics: A Canadian Perspective. *Canadian Journal of Infectious Diseases.* 14(2): 105-114.

Fairley,T.L., C.W. Kirkpatrick and J.E. Conn. 2005. Intragenomic heterogeneity in internal transcribed spacer rDNA in Neotropical malaria vector *Anopheles aquasalis* (Diptera: Culicidae). *Journal of Medical Entomology.* 42(5): 795-800.

Fairly, T.L, T.M. Renaud and J.E. Conn. 2000. Effects of local geographic barriers and latitude on population structure in *An. punctipennis* (Diptera: Culicidae). *Journal of Medical Entomology.* 37(5): 754-760.

Faran, M.E. 1979. The importance of an integrated approach in solving a problem in mosquito systematics. *Mosquito systematics.* 11(4): 280-288.

Floore, T.G., B.A. Harrison, and B.F. Eldridge. 1976. The *Anopheles (Anopheles) crucians* subgroup in the United States (Diptera: Culicidae). *Mosquito Systematics.* 8(1): 1-109.

Foley, D.H. and J.H. Bryan. 1991. *Anopheles annulipes* Walker (Diptera: Culicidae) at Griffith, New South Wales. 1. Two sibling species in sympatry. *Australian Journal of Entomology*: 109-112.

Foley, D.H., J.H. Bryan, D. Yeates, and A. Saul. 1998. Evolution and systematics of *Anopheles:* Insights from a molecular phylogeny of Australasian mosquitoes. *Molecular Phylogenetics and Evolution.* 9(2): 262-275.

Foley, D.H., R.C. Wilkerson, R.D. Cooper, M.E. Volovsek and J.H. Bryan. 2007. A molecular phylogeny of *Anopheles annulipes* (Diptera: Culicidae) *senso lato*: the most species-rich anopheline complex. *Molecular Phylogenetics and Evolution.* 43: 283-297.

Fritz, G.N., D. Dritz, T. Jensen, and R.K. Washino. 1991. Wing-scale pattern variation in *Anopheles punctipennis* (Say). *Mosquito systematics*. 23(3): 81-86.

Frizzi, G.1947. Salivary gland chromosomes of *Anopheles. Nature.* 160: 226-227.

Gimonneau, G., J. Bouyer, S. Morand, N.J. Besansky, A. Diabate, and F. Simard. 2010. A behavioural mechanism underlying ecological divergence in the malaria mosquito *Anopheles gambiae*. *Behavioral Ecology.* 21: 1087-1092.

Gimnig, J.E., M. Ombok, L. Kamau, and W.A. Hawley. 2001. Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya. *Journal of Medical Entomology.* 38(2): 282-288.

Green, C.A. and V. Baimai. 1985. Cytogenetic evidence for complex of species within the taxon *Anopheles maculatus* (Diptera: Culicidae). *Biological Journal of the Linnean Society*. 24: 321-328.

Hackett, B.J., J. Gimnig, W. Geulbeogo, C. Constantini, L.L Koekemoer, M. Coetzee, F.H. Collins and N.J. Besansky. 2000. Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *An. rivulorum*, and a uncover cryptic taxon. *Insect Molecular Biology*. 9(4): 369-374.

Hampston F.C and F.A. Lawson. 1967. The Mosquitoes of Colorado. Department of Health, Education and Welfare. Public Health Service. National Communicable Disease Center, Altanta, Georgia, USA. 140pp.

Harbach, R.E. 2004. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bulletin of Entomological Research*. 94: 537-553.

Harbach, R.E. and I.J. Kitching. 1998. Phylogeny and classification of the Culicidae (Diptera). *Systematic Entomology*.23: 327-370.

Harbach, R.E. and I.J. Kitching. 2005. Reconsideration of anopheline mosquito phylogeny (Diptera: Culicidae: Anophelinae) based on morphological data. *Systematics and Biodiversity*. 3(4): 345-374.

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R deWaard. 2003a. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, series B*. 270: 313-321.

Hebert P.D.N., S. Ratnasingham and J.R. deWaard. 2003b. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London, series B*. 270: S96-S99.

Hebert, P.D.N., E.H. Pentin, J.M. Burns, D.H. Janzen and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences*. 101(41): 14812-14817.

Hillis, D.M. and M.T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly Review of Biology*. 66(4): 411-453.

Horsfall, W.R., F.R. Voorhees and E.W. Cupp. 1970. Eggs of floodwater mosquitoes. XIII. Chorionic sculpturing. *Annals of the Entomological Society of America*. 63(6): 1709-1716.

Kampen, H. 2005. The ITS2 ribosomal DNA of *Anopheles beklemishevi* and further remarks on the phylogenetic relationships within the *Anopheles maculipennis* group of species (Diptera: Culicidae). *Parasitology Research.* 97: 118-128.

Kartman, L. 1953. Factors Influencing Infection of the Mosquito with Dirofilaria immitis (Leidy, 1856). *Experimental Parasitology.* 2: 27-78.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution.* 16: 111-120.

Kitzmiller. J.B. 1959. Race formation and speciation in mosquitoes. *Cold Spring Harbor Symposia on Qualitative Biology.* 24: 161-169.

Kitzmiller, J. B. and R. H. Baker. 1963. The salivary chromosomes of *Anopheles freeborni. Mosquito News.* 23: 254-261.

Kitzmiller, J. B. and R. H. Baker. 1965. The salivary chromosomes of *Anopheles earlei. Canadian Journal of Genetics and Cytology.* 7: 275-283.

Kitzmiller, J.B., D. Joslyn and R. D. Kreutzer. 1974. The salivary gland chromosomes of *Anopheles walkeri* Theobold. *Mosquito News.* 34: 22-28.

Klassen, W., French, W. L., Laven, H. and J. B. Kitzmiller. 1965. The salivary chromosomes of *Anopheles quadrimaculatus* Say. *Mosquito News.* 25: 328-334.

Kreutzer, R.D. and J.B. Kitzmiller. 1971a. Chromosomal similarity between *Anopheles perplexens* and *Anopheles punctipennis. Mosquito News.* 31(3): 409-417.

Kreutzer, R.D. and J.B. Kitzmiller. 1971b. Hybridization experiments between *Anopheles perplexens* and *Anopheles punctipennis. Journal of Heredity.* 61(4): 191-196.

Krzywinski, J. and N.J. Besanksy. 2003. Molecular systematics of *Anopheles*: From subgenera to subpopulations. *Annual Review of Entomology.* 48: 111-139.

Langerhans, R.B., M.E. Gifford, and E.O. Joseph. 2007. Ecological speciation in *Gambusia* fishes. *Evolution.* 61(9): 2056-2074.

LaRue, B., C. Gaudreau, H.O. Bagre, and G. Charpentier. 2009. Generalized structure and evolution of ITS1 and ITS1 rDNA in black flies (Diptera: Simuliidae). *Molecular Phylogenetics and Evolution.* 53: 749-757.

Levine, R.S., A.T. Peterson, and M.Q. Benedict. 2004. Distribution of members of the *Anopheles quadrimaculatus* Say s.l. (Diptera: Culicidae) and implications for their roles in malaria transmission in the United States. *Journal of Medical Entomology.* 41(4): 607-613.

Li, C. and R.C. Wilkerson. 2007. Intragenomic rDNA ITS2 variation in the Neotropical *Anopheles* ((Nyssorhynchus) *albitarsis* Complex (Diptera: Culicidae). *Journal of Heredity*. 98(1): 51-59.

Linley, J.R. and P.E. Kaiser. 1994. The eggs of *Anopheles punctipennis* and *Anopheles perplexens* (Diptera: Culicidae). *Mosquito systematics*. 26(2): 43-56.

Linley, J.R., P.E. Kaiser, and A.F. Cockburn. 1993. A description and morphometric study of the eggs of species of the *Anopheles quadrimaculatus* complex (Diptera: Culicidae). *Mosquito systematics*. 25(2): 124-147.

Linton, Y.-M., L. Smith, G. Koliopoulos, A. Samanidou-Voyadjoglou, A.K. Zounos and R.E. Harbach. 2003. Morphological and molecular characterization of *Anopheles (Anopheles) maculipennis* Meigen, type species of the genus and nominotypical member of the Maculipennis Complex. *Systematic Entomology*. 28: 39-55.

Lounibos, L.P, R.C. Wilkerson, J.E. Conn, L.J. Hribar, G.N Fritz, and J.A. Danoff-Burg. 1998. Morphological, molecular, and chromosomal discrimination of cryptic *Anopheles (Nyssorhynchus)* (Diptera: Culicidae) from South America. *Journal of Medical Entomology*. 35(5): 830-838.

Ludlow, C.S. 1907. Mosquito Notes. No. 5 continued. *The Canadian Entomologist*. 39: 266-268.

Lunt, D.H, D.-X. Zhang, J.M. Szymura, and G.M. Hewitt. 1996. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Molecular Biology*. 5(3): 153-165.

Manguin, S., D.R. Roberts, E.L. Peyton, E. Rejmankova, and J. Pecor. 1996. Characterization of *Anopheles pseudopunctipennis* larval habitats. *Journal of the American Mosquito Control Association*. 12(4): 619-626.

Marchi, A. and E. Pili. 1994. Ribosomal RNA genes in mosquitoes: localization by fluorescence *in situ* hybridization (FISH). *Heredity*. 72: 599-605.

Marinucci, M., R. Romi, P. Mancini, M. Di Luca and C. Severini. 1999. Phylogenetic relationships of seven palearctic members of the *maculipennis* complex inferred from ITS2 sequence data. *Insect Molecular Biology*. 8(4): 469-480.

McKinnon, J.S., S. Mori, B.K. Blackman, L. David, D.M. Kingsley, L. Jamieson, J. Chou, and D. Schluter. 2004. Evidence for ecology's role in speciation. *Nature*. 429: 294-298.

McLintock, J. and J. Iversen. 1975. Mosquitoes and human disease in Canada. *The Canadian Entomologist*. 107(7): 695-704.

Mitchell, A., F.A.H. Sperling and D.A. Hickey. 2002. Higher-level phylogeny of mosquitoes (Diptera: Culicidae): mtDNA data support a derived placement for Toxorhynchites. *Insect Systematics and Evolution.* 33(2): 163-174.

Munstermann, L.E. 1995. Mosquito Systematics: Current status, new trends, associated complications. *Journal of Vector Ecology.* 20(2): 129-138.

Munstermann, L.E. and J. E. Conn. 1997. Systematics of mosquito disease vectors (Diptera, Culicidae): Impact of molecular biology and cladistic analysis. *Annual Review of Entomology*. 42: 351-69.

Nicolescu, G., Y.-M. Linton, A. Vladimirescu, T.M Howard, and R.E. Harbach. 2004. Mosquitoes of the *Anopheles maculipennis* group (Diptera: Culicidae) in Romania, with the discovery and formal recognition of a new species based on morphological and molecular evidence. *Bulletin of Entomological Research.* 94: 525-535.

Onyabe, D.Y. and J.E. Conn. 1999. Intragenomic heterogeneity of a ribosomal DNA spacer (ITS2) varies regionally in the neotropical malaria vector *Anopheles nuneztovari* (Diptera: Culicidae). *Insect Molecular Biology*. 8(4): 435-442.

Pape, T. 1992. Cladistic analysis of mosquito chromosome data in *Anopheles* subgenus *Cellia* (Diptera: Culicidae). *Mosquito systematics.* 24(1): 1-11.

Paredes-Esquivel, C., M.J. Donnelly, R.E. Harbach and H. Townson. 2009. A molecular phylogeny of mosquitoes in the *Anopheles barbirostris* Subgroup reveals cryptic species: Implications for identification of disease vectors. *Molecular Phylogenetics and Evolution.* 50: 141-151.

Paskewitz, S.M. D.M. Wesson, and F. H. Collins. 1993. The internal transcribed spacers of ribosomal DNA in five members of the *Anopheles gambiae* species complex. *Insect Molecular Biology*. 2(4): 247-257.

Paterson, H.E. 1962. Status of the East African salt-water-breeding variant of *Anopheles gambiae* Giles. *Nature.* 195: 469-470.

Peters, H.T. 1943. Studies on the biology of *Anopheles walkeri* Theobold (Diptera: Culicidae). *The Journal of Parasitology*. 29(2): 117-122.

Peterson, J.J., H.C. Chapman, and O.R. Wells. 1969. Predation of *Anopheles barberi* Coquillett on first instar mosquito larvae. *Mosquito News.* 29(1): 134-135.

Phan, V.T., N.D. Manh, T.D. Hinh, and N.T. Vien. 1990. *Anopheles* (*Anopheles*) *cucphuongensis*: a new species from Vietnam (Diptera: Culicidae). *Mosquito Systematics.* 22(3): 145-148.

Porter, C.H. and F.H. Collins. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *American Journal of Tropical Medicine and Hygiene.* 45(2): 271-279.

Porter, C.H. and F.H. Collins. 1996. Phylogeny of the Nearctic members of the *Anopheles maculipennis* species group derived from the D2 variable region of 28S ribosomal RNA. *Molecular Phylogenetics and Evolution.* 6(2): 178-188.

Pratt, H.D. 1952. Notes on *Anopheles earlei* and other American species of the *Anopheles maculipennis* complex. *American Journal of Tropical Medicine and Hygiene.* 1(3): 484-493.

Proft, J., W.A. Maier and H. Kampen. 1999. Identification of six sibling species of the *Anopheles maculipennis* complex (Diptera: Culicidae) by a polymerase chain reaction assay. *Parasitology Research.* 85: 837-843.

Ramirez, C.C.L. and E.M.B. Desson. 2000. Chromosomal evidence for sibling species of the malaria vector *Anopheles cruzii. Genome.* 43: 143-151.

Reidenbach, K.R., S. Cook, M.A. Bertone, R.E. Harbach, B.M. Wiegmann, and N. Besansky. 2009. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. *BMC Evolutionary Biology.* 9: 298.

Reinert, J.F. 2010. List of anophelines species with published illustrations and/or descriptions of eggs (Diptera: Culicidae: Anophelinae). *European Mosquito Bulletin.* 28: 103-142.

Reinert, J.E., P.E. Kaiser and J.A. Seawright. 1997. Analysis of the *Anopheles (Anopheles) quadrimaculatus* complex of sibling species (Diptera: Culicidae) using morphological, cytological, molecular, genetic, biochemical, and ecological techniques in an integrated approach. *Journal of the American Mosquito Control Association.* 13: 1-102.

Rejmankova, E., D.R. Roberts, R.E. Harbach, J. Pecor, E.L. Peyton, S. Mangiun, R. Krieg, J. Polanco, and L. Legters. 1993. Environmental and regional determinants of *Anopheles* (Diptera: Culicidae) larval distribution in Belize, Central America. *Environmental Entomology.* 22(5): 978-992.

Rempel, J.G. 1950. A guide to the mosquito larvae of western Canada. *Canadian Journal of Research D.* 28: 207-248.

Reschke, C., R. Reid, J. Jones, T. Feeney, and H. Potter (Alvar Working Group). 1999. Conserving Great Lakes Alvars. Final Technical Report of the International Alvar Conservation Initiative. The Nature Conservancy, Great Lakes Program. Chicago, Illinois. 241pp.

Ritchie, A., A. Blackwell, G. Malloch, and B. Fenton. 2004. Heterogeneity of ITS1 sequences in the biting midge *Culicoides impunctatus* (Goetghebuer) suggests a population in Argyll, Scotland may be genetically distinct. *Genome.* 47: 546-558.

Rivera, J. and D.C. Currie. 2009. Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). *Molecular Ecology Resources.* 9 (Suppl. 1): 224-236.

Roe, A.D. and F.A.H. Sperling. 2007. Patterns of evolution of mitochondrial cytochrome *c* oxidase I and II DNA and implications for DNA barcoding. *Molecular Phylogenetics and Evolution.* 44: 325-345.

Sáez, A.G. and E. Lozano. 2005. Body doubles. *Nature.* 433: 111.

Sallum, M.A.M., P.G. Foster, C. Li, R. Sithiprasasna, and R.C. Wilkerson. 2007. Phylogeny of the Leucosphyrus Group of *Anopheles* (*Cellia*) (Diptera: Culicidae) based on mitochondrial gene sequences. *Annals of the Entomological Society of America.* 100(1): 27-35.

Sallum, M.A.M., T.R. Schultz, P.G. Foster, K. Aronstein, R.A. Wirtz, and R.C. Wilkerson. 2002. Phylogeny of Anophelinae (Diptera: Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. *Systematic Entomology.* 27: 361-382.

Sames, W.J., A. Duffy, F.A. Maloney, J.S. Townzen, J.M. Brauner, C.P. McHugh, and J. Lilja. 2007. Distribution of mosquitoes in Washington State. *Journal of the American Mosquito Control Association.* 23(4): 442-448.

Schlick-Steiner, B.C., B. Seifert, C. Stauffer, E. Christian, R.H. Crozier, and F.M. Steiner. 2007. Without morphology, cryptic species stay in taxonomic crypsis following discovery. *Trends in Ecology and Evolution.* 22(8): 391-392.

Schluter, D. 2001. Ecology and the origin of species. *Trends in Ecology and Evolution.* 16(7): 372-380.

Schluter, D. 2009. Evidence for ecological speciation and its alternative. *Science.* 323: 737-741.

Service, M. 1964. An analysis of individual and seasonal variations occurring in the wing ornamentation of *Anopheles* (*Cellia*) *funestus* Giles (Diptera: Culicidae). *Proceeding of the Royal Entomological Society of London B.* 33: 139-164.

Sharpe, R.G., R.E. Harbach, and R.K. Butlin. 2000. Molecular variation and phylogeny of members of the Minimus Group of *Anopheles* subgenus *Cellia. Systematic Entomology.* 25(2): 263-272.

Sheffield, C.S., P.D.N. Hebert, P.G. Kevan, and L. Packer. 2009. DNA barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies. *Molecular Ecology Resources.* 9 (Suppl. 1): 196-207.

Shililu, J., T. Ghembremeskel, F. Seulu, S. Mengistu, H. Fekadu, M. Zerom, A. Ghebregziabiher, D. Sintasath, G. Bretas, C. Mbogo, J. Githure, E. Brantly, R. Novak, and J. C. Beier. 2003. Larval habitat diversity and ecology of anopheline larvae in Eritrea. *Journal of Medical Entomology.* 40(6): 921-929.

Shipp, J.L. R.E. Wright, and D.H. Pengelly. 1978. Distribution of *Aedes triseriatus* (Say) and *Aedes hendersoni* Cockerell in southwestern Ontario, 1975-76. *Mosquito News.* 38(3): 408-412.

Siler, C.D., A.C. Diesmos, C. W. Linkem, M.L. Diesmos, and R.M. Brown. 2010. A new species of limestone-forest frog, genus *Platymantis* (Amphibia: Anura: Ceratobatrachudae) from central Luzon Island, Philippines. *Zootaxa.* 2482: 49-63.

Simon C., F. Frati, A. Beckenbach, B. Crespi, H. Liu and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of Entomological Society of America.* 87: 651-701.

Smith, M.A., B.L. Fisher, and P.D.N. Hebert. 2005. DNA barcoding for effective diversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences.* 360: 1825-1834.

Smith, M.A., D.M Wood, D.H. Janzen, W. Hallwachs, and P.D.N. Hebert. 2007. DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proceedings of the National Academy of Sciences.* 104(12): 4967-4972.

Smith, S.M. and R.M. Trimble. 1973. The biology of tree-holes of Point Pelee National Park, Ontario. I. New mosquitoes records for Canada (Diptera: Culicidae). *The Canadian Entomologist.* 105: 1585-1586.

Tamura, K, J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution.* 24: 1596-1599.

Thielman, A. and F.F. Hunter. 2007. Photographic key to the adult female mosquitoes of Canada. *Canadian Journal of Arthropod Identification.* CJAI 04.

Thielman, A. and F. F. Hunter. 2010. General Status Summary for the Mosquitoes of Canada. Wild Species 2010 Report (www.wildspecies.ca/rpts.cfm?lang=e). Prepared for the National General Status Working Group (NGSWG).

Turell, M.J., M.L. O'Guinn, D.J. Dohm, and J.W. Jones. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for the West Nile virus. *Journal of Medical Entomology.* 38(2): 130-134.

Walton, C., J.M. Handley, C. Kuvangkadilok, F.H. Collins, R.E. Harbach, V. Raimai and R.K. Butlin. 1999a. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. *Medical and Veterinary Entomology.* 13: 24-32.

Walton, C., R.G. Sharpe, S.J. Pritchard, N.J. Thelwell and R.K. Butlin. 1999b. Molecular identification of mosquito species. *Biological Journal of the Linnean Society.* 68: 241-256.

Walton, C., P. Somboon, R.E. Harbach, S. Zhang, I. Weerasinghe, S.M. O'Loughlin, S. Phompida, T. Sochantha, W. Tun-Lin, B. Chen, and R.K. Butlin. 2007a. Molecular identification of mosquito species in the *Anopheles annularis* group in southern Asia. *Medical and Veterinary Entomology.* 21: 30-35.

Walton, C., P. Somboon, S.M. O'Loughlin, S. Zhang, R.E. Harbach, Y.-M. Linton, B. Chen, K. Nolan, S. Duong, M.-Y. Fong, I. Vythilingum, Z.D. Mohammed, Ho Dinh Trung, and R.K. Butlin. 2007b. Genetic diversity and molecular identification of mosquito species in the *Anopheles maculatus* group using the ITS2 region of rDNA. *Infection, Genetics and Evolution.* 7: 93-102.

Wesson, D.M., C.H. Porter, and F.H. Collins. 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). *Molecular Phylogenetics and Evolution.* 1(4): 253-269.

White, G.B. 1977. The place of morphological studies in the investigation of *Anopheles* species complexes. *Mosquito Systematics.* 9(1): 1-24.

White, G.D. 1978. Systematic reappraisal of the *Anopheles maculipennis* complex. *Mosquito Systematics.* 10(1): 13-44.

Wilkerson, R.C., P.G. Foster, C. Li, and M.A.M. Sallum. 2005. Molecular phylogeny of Neotropical *Anopheles* (*Nyssorhynchus*) *albitarsis* Species Complex (Diptera: Culicidae). *Annals of the Entomological Society of America.* 98(6): 918-925.

Wilkerson, R.C., J.F. Reinert, and C. Li. 2004. Ribosomal DNA ITS2 sequences differentiate six species on the *Anopheles crucians* Complex (Diptera: Culicidae). *Journal of Medical Entomology.* 41(3): 392-401.

Williams, H.C., S.J. Ormond, and M.W. Bruford. 2006. Molecular systematics and phylogeography of the cryptic species complex *Baetis rhodani* (Ephemeroptera, Baetidae). *Molecular Phylogenetics and Evolution.* 40: 370-382.

World Health Organization. Malaria Report 2009. (http://www.who.int/malaria/world_malaria_report_2009/en/index.html)

Wood, D.M., P.T. Dang, and R.A. Ellis. 1979. The Insect and Arachnids of Canada. Part VI. The Mosquitoes of Canada. National Research Council Publishers, Ottawa, Canada. 360pp.

Zavortink, T.J. 1990. Classical taxonomy of mosquitoes – a memorial to John N. Belkin. *Journal of the American Mosquito Control Association.* 6(4): 593-601.

Zucker, J.R. 1996. Changing patterns of autochthonous malaria transmission in the United States: a review of recent out-breaks. *Emerging Infectious Diseases.* 2(1): 37–43.

Appendix I

Abbreviations of sampling regions from which *Anopheles* specimens were collected during 2005-2009 field seasons.

Region	Code
Algonquin Provincial Park, ON	AL
Southern British Columbia	BC
Bruce Peninsula, ON	BP
First Nations, Caledonia, ON	FN
Halton, ON	HL
Niagara Region, ON	NI
Island of Newfoundland, NL	NF
Manitoulin Island and area, ON	NO
Ottawa Region, ON	OT
Radisson, Quebec	RQ
Windsor Essex County, ON	WE

Appendix II

Anopheles mosquito collection database

An online database was designed by a computer programmer (Ming Lin) at Entomogen, Inc. (Dr. Fiona Hunter, President) to maintain all collection information associated with all mosquito collections conducted during the 2005-2009 field seasons. Raw data were entered into logs by collection method (i.e., larval dipping collections, adult CDC light trappings, adult landing aspirations, and oviposition experiments), and can be accessed using different queries that were built into the database that allow searching and summarizing data by species, region, data collected, etc. Query results can be exported to Excel for data analysis. Two examples of database functions follow: (1) ecological data associated with larval dipping collections (Table 1) are in the database, and accessible under the Larval Dipping Log; and (2) a search for *An. walkeri* larvae using the Larval ID query produces a table that includes the collection type, site code, date collected, preservation method, number of individuals, and storage location (Box and Slot) and can be exported to Excel (Table 2).

Table 1. Larval habitat data recorded for mosquito-positive larval dipping collections.

Data Type	Description
Code	unique identifier for each collection
Date of Collection	day/month/year
Location	Street address, city, province
Latitdue	ex. 42.3582 (decimal format)
Longitude	ex. -79.2850 (decimal format)
Description	general description of environment
Air Temperature	in degrees Celsius
Water Temperature	in degrees Celsius
Sky	clear, partly cloudy, or overcast
Windy	yes, no
Container Type	natural or artificial
Bottom	mud, silt, stone, etc.
Depth	<10cm, 10-50cm, >50cm
Diameter	<1m, 1-10m, 10-50m, >50m
Shade	full sun, partial shade, full shade
Emergent Vegetation	present/absent
EV Type (if present)	list of types
Floating Vegetation	present/absent
FV Type (if present)	list of types
Container Type	one of 13 classifications (Table 4.3)

Table 2. Excel file table produced by database query (Mosquito ID – Larvae) of *An. walkeri* larvae collected from all possible regions and years of collection.

Collection Type	Code	Date Collected	Species	Preservation	Num	Box	Slot
Larval Dipping	lWE13	13-Jun-05	An. walkeri	Morphological	2	L1	55
Larval Dipping	lWE13	13-Jun-05	An. walkeri	Cytogenetic	2	L1	54
Larval Dipping	lNO6	14-Jul-05	An. walkeri	Morphological	1	L3	1
Larval Dipping	lNO6	14-Jul-05	An. walkeri	Cytogenetic	3	L3	10
Larval Dipping	lNO6	14-Jul-05	An. walkeri	Molecular	1	L3	38
Larval Dipping	lNO7	14-Jul-05	An. walkeri	Molecular	1	L3	22
Larval Dipping	lNO7	14-Jul-05	An. walkeri	Molecular	1	25	64
Larval Dipping	lNO13	15-Jul-05	An. walkeri	Morphological	4	L3	35
Larval Dipping	lNO13	15-Jul-05	An. walkeri	Molecular	1	ML1	38
Larval Dipping	lNO13	15-Jul-05	An. walkeri	Molecular	1	ML1	39
Larval Dipping	lNO13	15-Jul-05	An. walkeri	Cytogenetic	4	L2	97
Larval Dipping	lNO9	15-Jul-05	An. walkeri	Cytogenetic	1	L3	15
Larval Dipping	lNO14	16-Jul-05	An. walkeri	Cytogenetic	1	L3	33
Larval Dipping	lNO14	16-Jul-05	An. walkeri	Cytogenetic	1	L3	29
Larval Dipping	lNI16	28-Jul-05	An. walkeri	Cytogenetic	1	L3	71
Larval Dipping	lAL20	01-Aug-05	An. walkeri	Morphological	1	L3	78
Larval Dipping	lNI22	16-Aug-05	An. walkeri	Morphological	2	L3	63
Larval Dipping	lNI23	16-Aug-05	An. walkeri	Molecular	1	ML1	40
Larval Dipping	lNO39	04-Aug-06	An. walkeri	Live Dissections	1		
Larval Dipping	lNO39	04-Aug-06	An. walkeri	Molecular	1	L7	45
Larval Dipping	lNI78	06-Sep-07	An. walkeri	Molecular	1	L10	78
Larval Dipping	lWE104	12-Jun-08	An. walkeri	Molecular	1	ML2	3
Larval Dipping	lWE122	14-Jun-08	An. walkeri	Morphological	2	L12	14
Larval Dipping	lBP09	20-Jun-08	An. walkeri	Morphological	1	L12	19

The database, as well as preserved larval and adults specimens, have been deposited in the Canadian National Collection of Insects, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada. The database can be accessed at: http://www.canacoll.org/Diptera/Aynsley/.

Appendix III

Collection data associated with individuals for which COI, ITS2, or ITS1 sequences were obtained (see Chapter Three). Latitude and longitude units: Decimal degrees (WGS84).

Code	Species	Date	Region	City	Stage	Latitude	Longitude
lNI76-1	*An. quadrimaculatus s.l.*	29-Aug-07	NI	Smithville	adult	43.0912	-79.53668333
lNI85-1	*An. quadrimaculatus s.l.*	12-Sep-07	NI	Pelham	adult	43.09933333	-79.30981667
lNI60-2	*An. quadrimaculatus s.l.*	17-Aug-07	NI	Pelham	adult	43.102	-79.30375
lNI76-2	*An. quadrimaculatus s.l.*	29-Aug-07	NI	Smithville	adult	43.0912	-79.53668333
lNI76-3	*An. quadrimaculatus s.l.*	29-Aug-07	NI	Smithville	adult	43.0912	-79.53668333
lNI90-5	*An. quadrimaculatus s.l.*	19-Sep-07	NI	St. Catharines	adult	43.11008334	-79.2645
lNI84-1	*An. smaragdinus*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI84-2	*An. smaragdinus*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI73-2	*An. smaragdinus*	28-Aug-07	NI	Wainfleet	adult	42.88578333	-79.27893333
lNI73-3	*An. smaragdinus*	28-Aug-07	NI	Wainfleet	adult	42.88578333	-79.27893333
lNI73-5	*An. smaragdinus*	28-Aug-07	NI	Wainfleet	adult	42.88578333	-79.27893333
lNI94-12	*An. smaragdinus*	8-Oct-07	NI	Virgil	adult	43.24886667	-79.12631667
lNI92-10	*An. punctipennis*	19-Sep-07	NI	Pelham	adult	43.09598334	-79.33191667
lNI92-11	*An. punctipennis*	19-Sep-07	NI	Pelham	adult	43.09598334	-79.33191667
lNI94-14	*An. punctipennis*	8-Oct-07	NI	Virgil	adult	43.24886667	-79.12631667
lNI94-15	*An. punctipennis*	8-Oct-07	NI	Virgil	adult	43.24886667	-79.12631667
lNI93-3	*An. punctipennis*	19-Sep-07	NI	St. Ann's	adult	43.03558334	-79.4708
lNI93-4	*An. punctipennis*	19-Sep-07	NI	St. Ann's	adult	43.03558334	-79.4708
lNI92-7	*An. perplexens*	19-Sep-07	NI	Pelham	adult	43.09598334	-79.33191667
lFN1-1	*An. perplexens*	19-Aug-06	NI	Caledonia	adult	43.049322	-80.06218
lNI84-3	*An. perplexens*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI92-8	*An. perplexens*	19-Sep-07	NI	Pelham	adult	43.09598334	-79.33191667
lNI91-9	*An. perplexens*	19-Sep-07	NI	Pelham	adult	43.0975	-79.33141667
lNI89-4	*An. perplexens*	14-Sep-07	NI	St. Catharines	adult	43.10493334	-79.2871
lNO8-6	*An. earlei*	15-Jul-05	NO	Manitoulin	adult	45.80194	-82.115
lNO5-2	*An. earlei*	14-Jul-05	NO	Manitoulin	adult	45.67694	-82.26694
lNO5-3	*An. earlei*	14-Jul-05	NO	Manitoulin	adult	45.67694	-82.26694
lNO49-1	*An. earlei*	5-Aug-06	NO	Manitoulin	adult	45.80528	-82.1975
lNO5-4	*An. earlei*	14-Jul-05	NO	Manitoulin	adult	45.67694	-82.26694
lNO5-5	*An. earlei*	14-Jul-05	NO	Manitoulin	adult	45.67694	-82.26694
lBC55-7	*An. freeborni*	20-Sep-05	BC	Kamloops	adult	50.85333	-120.3075
lBC24-1	*An. freeborni*	24-Jun-05	BC	Vernon	adult	50.36056	-119.28083
lBC55-8	*An. freeborni*	20-Sep-05	BC	Kamloops	adult	50.85333	-120.3075
lOT3-5	*An. perplexens*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-6	*An. perplexens*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-7	*An. perplexens*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lNO7-3	*An. walkeri*	14-Jul-05	NO	Manitoulin	adult	45.84361	-82.20194
lNO13-5	*An. walkeri*	15-Jul-05	NO	Manitoulin	adult	45.80361	-81.98361
lNO13-6	*An. walkeri*	15-Jul-05	NO	Manitoulin	adult	45.80361	-81.98361
lNO13-7	*An. walkeri*	15-Jul-05	NO	Manitoulin	adult	45.80361	-81.98361
lNO13-8	*An. walkeri*	15-Jul-05	NO	Manitoulin	adult	45.80361	-81.98361
lNO11-3	*An. walkeri*	15-Jul-05	NO	Manitoulin	adult	45.78472	-81.94333
NS004-10	*An. barberi*	27-Jul-06	NI	Niagara Falls	adult	43.0623	-79.1743
NS006-10	*An. barberi*	8-Sep-06	NI	Niagara Falls	larva	43.0623	-79.1743
NH009-1	*An. barberi*	27-Jul-06	NI	Niagara Falls	larva	43.0623	-79.1743
NS003-10	*An. barberi*	8-Sep-06	NI	Niagara Falls	larva	43.0623	-79.1743
NS007-10	*An. barberi*	27-Jul-06	NI	Niagara Falls	larva	43.0623	-79.1743

Appendix III (con't)

Code	Species	Date	Region	City	Stage	Latitude	Longitude
lNI80-5	*An. quadrimaculatus s.l.*	5-Sep-07	NI	Virgil	adult	43.24825	-79.1263
lNI68-1	*An. quadrimaculatus s.l.*	21-Aug-07	NI	Ridgeville	adult	43.0282	-79.30981666
lNI94-8	*An. quadrimaculatus s.l.*	8-Oct-07	NI	Virgil	adult	43.24886667	-79.12631667
lNI85-3	*An. quadrimaculatus s.l.*	12-Sep-07	NI	Pelham	adult	43.09933333	-79.30981667
lNI72-1	*An. quadrimaculatus s.l.*	28-Aug-07	NI	Wainfleet	adult	42.88606667	-79.32575
lNI60-5	*An. quadrimaculatus s.l.*	17-Aug-07	NI	Pelham	adult	43.102	-79.30375
lNI78-2	*An. quadrimaculatus s.l.*	6-Sep-07	NI	Queenston	adult	43.15116667	-79.08425
lNI80-21	*An. quadrimaculatus s.l.*	5-Sep-07	NI	Virgil	adult	43.24825	-79.1263
lNI82-8	*An. quadrimaculatus s.l.*	7-Sep-07	NI	St. Catharines	adult	43.13968333	-79.2999
lNI78-4	*An. quadrimaculatus s.l.*	6-Sep-07	NI	St. Ann's	adult	43.15116667	-79.08425
lNI77-1	*An. quadrimaculatus s.l.*	29-Aug-07	NI	St. Ann's	adult	43.0355	-79.46913333
lNI60-1	*An. smaragdinus*	17-Aug-07	NI	Pelham	adult	43.102	-79.30375
lNI60-3	*An. smaragdinus*	17-Aug-07	NI	Pelham	adult	43.102	-79.30375
lNI80-1	*An. smaragdinus*	5-Sep-07	NI	Virgil	adult	43.24825	-79.1263
lNI80-2	*An. smaragdinus*	5-Sep-07	NI	Virgil	adult	43.24825	-79.1263
lNI80-4	*An. smaragdinus*	5-Sep-07	NI	Virgil	adult	43.24825	-79.1263
lNI73-4	*An. smaragdinus*	28-Aug-07	NI	Beamsvile	adult	43.15385	-79.48783333
lNI91-8	*An. diluvialis*	19-Sep-07	NI	Pelham	adult	43.0975	-79.33141667
lNI81-1	*An. inundatus*	7-Sep-07	NI	St. Catharines	adult	43.13968333	-79.2999
lOT3-10	*An. earlei*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-11	*An. earlei*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-12	*An. earlei*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-13	*An. earlei*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-14	*An. earlei*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lOT3-15	*An. earlei*	23-Aug-05	OT	Kaladar	adult	44.76611	-76.7575
lAL21-7	*An. earlei*	1-Aug-05	AL	Algonquin	larva	45.57694	-78.44889
lAL21-8	*An. earlei*	1-Aug-05	AL	Algonquin	larva	45.57694	-78.44889
lAL22-10	*An. earlei*	2-Aug-05	AL	Algonquin	larva	45.58028	-78.39778
lAL22-11	*An. earlei*	2-Aug-05	AL	Algonquin	larva	45.58028	-78.39778
lOT3-16	*An. earlei*	23-Aug-05	OT	Kaladar	larva	44.76611	-76.7575
lOT3-17	*An. earlei*	23-Aug-05	OT	Kaladar	larva	44.76611	-76.7575
lBC57-5	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-6	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-7	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-8	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-9	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-10	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-11	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-12	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-13	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-14	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC58-1	*An. punctipennis*	27-Jun-08	BC	Chilliwack	adult	49.2	-121.7333
lBC58-2	*An. punctipennis*	27-Jun-08	BC	Kamloops	adult	49.2	-121.7333
lBC57-15	*An. punctipennis*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-16	*An. punctipennis*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-17	*An. punctipennis*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-18	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-19	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-20	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-21	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-22	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC12-1	*An. earlei*	22-Jun-05	BC	Kamloops	adult	50.67889	-120.3025
lBC16-1	*An. earlei*	23-Jun-05	BC	Kamloops	adult	51.01833	-120.22806
lBC44-1	*An. earlei*	27-Jun-05	BC	Grand Forks	adult	49.15417	-118.54583
lBC55-6	*An. earlei*	20-Sep-05	BC	Kamloops	adult	50.85333	-120.3075

Appendix III (con't)

Code	Species	Date	Region	City	Stage	Latitude	Longitude
lBC57-7	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-8	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-9	*An. freeborni*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-10	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-11	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-12	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-13	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-14	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC58-1	*An. punctipennis*	27-Jun-08	BC	Chilliwack	adult	49.2	-121.7333
lBC58-2	*An. punctipennis*	27-Jun-08	BC	Kamloops	adult	49.2	-121.7333
lBC57-15	*An. punctipennis*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-16	*An. punctipennis*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-17	*An. punctipennis*	18-Aug-08	BC	Kamloops	adult	50.852	120.2978
lBC57-18	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-19	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-20	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-21	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-22	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC12-1	*An. earlei*	22-Jun-05	BC	Kamloops	adult	50.67889	-120.3025
lBC16-1	*An. earlei*	23-Jun-05	BC	Kamloops	adult	51.01833	-120.22806
lBC44-1	*An. earlei*	27-Jun-05	BC	Grand Forks	adult	49.15417	-118.54583
lBC55-6	*An. earlei*	20-Sep-05	BC	Kamloops	adult	50.85333	-120.3075
lBC55-9	*An. earlei*	20-Sep-05	BC	Kamloops	adult	50.85333	-120.3075
lBC55-10	*An. earlei*	20-Sep-05	BC	Kamloops	larva	50.85333	-120.3075
lBC55-11	*An. earlei*	20-Sep-05	BC	Kamloops	larva	50.85333	-120.3075
lBC55-12	*An. earlei*	20-Sep-05	BC	Kamloops	larva	50.85333	-120.3075
lBC58-6a	*An. earlei*	27-Jun-08	BC	Chilliwack	larva	49.2	-121.7333
lBC58-7a	*An. earlei*	27-Jun-08	BC	Chilliwack	larva	49.2	-121.7333
lBC58-4	*An. punctipennis*	27-Jun-08	BC	Chilliwack	larva	49.2	-121.7333
lBC57-3	*An. earlei*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-4	*An. earlei*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lNI84-4	*An. punctipennis*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI84-5	*An. punctipennis*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI84-6	*An. punctipennis*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI84-7	*An. punctipennis*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI84-8	*An. punctipennis*	7-Sep-07	NI	Welland	adult	42.98073333	79.2057
lNI64-6	*An. quadrimaculatus s.l.*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
lNI64-7	*An. quadrimaculatus s.l.*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
lNI64-8	*An. quadrimaculatus s.l.*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
lNI64-9	*An. quadrimaculatus s.l.*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
lNI64-10	*An. quadrimaculatus s.l.*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
lNI26-1	*An. perplexens*	9-Sep-05	NI	Wainfleet	adult	42.89722	-79.32639
lNI26-2	*An. perplexens*	9-Sep-05	NI	Wainfleet	adult	42.89722	-79.32639
lNI26-3	*An. perplexens*	9-Sep-05	NI	Wainfleet	adult	42.89722	-79.32639
lNI26-4	*An. perplexens*	9-Sep-05	NI	Wainfleet	adult	42.89722	-79.32639
lNI26-5	*An. perplexens*	9-Sep-05	NI	Wainfleet	adult	42.89722	-79.32639
lAL22-4	*An. earlei*	2-Aug-05	AL	Algonquin	adult	45.58028	-78.39778
lAL21-4	*An. earlei*	1-Aug-05	AL	Algonquin	adult	45.57694	-78.44889
lAL21-5	*An. earlei*	1-Aug-05	AL	Algonquin	adult	45.57694	-78.44889
lAL20-1	*An. earlei*	1-Aug-05	AL	Algonquin	adult	45.58083	-78.51611
lAL21-6	*An. earlei*	1-Aug-05	AL	Algonquin	adult	45.57694	-78.44889
NI136-4a	*An. punctipennis*	31-Jul-08	NI	Port Colbourne	larva	42.9294	-79.255
lOT8-10	*An. quadrimaculatus s.l.*	24-Aug-05	OT	Perth	larva	44.87361	-76.20667
lNI18-1	*An. quadrimaculatus s.l.*	8-Jun-05	NI	Virgil	larva	43.19545	-79.18523

Appendix III (con't)

Code	Species	Date	Region	City	Stage	Latitude	Longitude
LP06-2a	*An. punctipennis*	27-Aug-08	LP	Abottsford	adult	42.8092	-80.6325
LP06-2b	*An. punctipennis*	27-Aug-08	LP	Abottsford	adult	42.8092	-80.6325
LP02-7	*An. walkeri*	26-Aug-08	LP	Long Point	adult	42.5799	-80.3783
LP02-8	*An. walkeri*	26-Aug-08	LP	Long Point	adult	42.5799	-80.3783
lNO16-3	*An. punctipennis*	16-Jul-05	NO	McKerrow	adult	46.29639	-81.75139
lNO16-4	*An. punctipennis*	16-Jul-05	NO	McKerrow	adult	46.29639	-81.75139
lWE29-10	*An. quadrimaculatus s.l.*	19-Sep-05	WE	Windsor	adult	42.33167	-82.92639
lWE29-11	*An. quadrimaculatus s.l.*	19-Sep-05	WE	Windsor	adult	42.33167	-82.92639
lNI58-4	*An. quadrimaculatus s.l.*	15-Aug-07	NI	Wainfleet	adult	42.88111667	-79.30451666
lNI58-5	*An. quadrimaculatus s.l.*	15-Aug-07	NI	Wainfleet	adult	42.88111667	-79.30451666
lNI65-1	*An. punctipennis*	21-Aug-07	NI	Queenston	adult	43.16498333	-79.1336
lNI65-2	*An. punctipennis*	21-Aug-07	NI	Queenston	adult	43.16498333	-79.1336
lNI74-1	*An. quadrimaculatus s.l.*	29-Aug-07	NI	Beamsvile	adult	43.15385	-79.48783333
lNI74-2	*An. quadrimaculatus s.l.*	29-Aug-07	NI	Beamsvile	adult	43.15385	-79.48783333
lBC2-6	*An. punctipennis*	21-Jun-05	BC	Chilliwack	adult	49.187601	-121.744137
lBC2-7	*An. punctipennis*	21-Jun-05	BC	Chilliwack	adult	49.187601	-121.744137
lNI92-1	*An. punctipennis*	19-Sep-07	NI	Pelham	larva	43.09598334	-79.33191667
lNI80-6	*An. quadrimaculatus s.l.*	5-Sep-07	NI	Virgil	larva	43.24825	-79.1263
lNO7-4	*An. walkeri*	14-Jul-05	NO	Manitoulin	larva	45.84361	-82.20194
lBC57-25	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-26	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-27	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-28	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lBC57-29	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
lNO39-3	*An. earlei*	4-Aug-06	NO	Manitoulin	larva	45.74722	-82.17361
lNO40-3	*An. earlei*	4-Aug-06	NO	Manitoulin	larva	45.71333	-82.21306
lNO40-4	*An. earlei*	4-Aug-06	NO	Manitoulin	larva	45.71333	-82.21306
lNO40-5	*An. earlei*	4-Aug-06	NO	Manitoulin	larva	45.71333	-82.21306
lNO48-1	*An. earlei*	6-Aug-06	NO	Manitoulin	larva	45.87472	-82.44222
lNO13-9	*An. walkeri*	15-Jul-05	NO	Manitoulin	larva	45.80361	-81.98361
lNO13-10	*An. walkeri*	15-Jul-05	NO	Manitoulin	larva	45.80361	-81.98361
LP01-10	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
LP01-11	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
LP01-12	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
lNI225-13	*An. barberi*	27-Sep-09	NI	Niagara Falls	female	43.0625	-79.1745
lNI225-14	*An. barberi*	27-Sep-09	NI	Niagara Falls	female	43.0625	-79.1745
lNI225-15	*An. barberi*	27-Sep-09	NI	Niagara Falls	female	43.0625	-79.1745
lNI225-16	*An. barberi*	27-Sep-09	NI	Niagara Falls	male	43.0625	-79.1745
lNI225-17	*An. barberi*	27-Sep-09	NI	Niagara Falls	male	43.0625	-79.1745
lNI226-1	*An. perplexens*	27-Sep-09	NI	St. Catharines	female	43.1095	-79.2646
lNI226-2	*An. perplexens*	27-Sep-09	NI	St. Catharines	female	43.1095	-79.2646
lNI226-3	*An. perplexens*	27-Sep-09	NI	St. Catharines	female	43.1095	-79.2646
lNI226-4	*An. perplexens*	27-Sep-09	NI	St. Catharines	female	43.1095	-79.2646
lNI23-1	*An. walkeri*	16-Aug-05	NI	Stevensville	larva	42.94861	-79.05167
lNI68-7	*Cx. territans*	21-Aug-07	NI	Ridgeville	male	43.0282	-79.30981666
lNI68-8	*Cx. territans*	21-Aug-07	NI	Ridgeville	male	43.0282	-79.30981666
LP01-13	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
LP01-14	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
lNI91-1	*An. punctipennis*	19-Sep-07	NI	Pelham	larva	43.0975	-79.33141667
lNI91-2	*An. punctipennis*	19-Sep-07	NI	Pelham	larva	43.0975	-79.33141667
lNI91-3	*An. punctipennis*	19-Sep-07	NI	Pelham	larva	43.0975	-79.33141667
lNI89-2	*An. punctipennis*	14-Sep-07	NI	St. Catharines	larva	43.10493334	-79.2871
lNI89-3	*An. punctipennis*	14-Sep-07	NI	St. Catharines	larva	43.10493334	-79.2871
lNI92-3	*An. perplexens*	19-Sep-07	NI	St. Ann's	larva	43.09598334	-79.33191667

Appendix III (con't)

Code	Species	Date	Region	City	Stage	Latitude	Longitude
INI92-5	*An. perplexens*	19-Sep-07	NI	St. Ann's	larva	43.09598334	-79.33191667
INI91-5	*An. perplexens*	19-Sep-07	NI	Pelham	larva	43.0975	-79.33141667
INI89-1	*An. perplexens*	14-Sep-07	NI	St. Catharines	larva	43.10493334	-79.2871
INI91-7	*An. quadrimaculatus s.l.*	19/09/1007	NI	Pelham	larva	43.0975	-79.33141667
INI90-2	*An. quadrimaculatus s.l.*	19-Sep-07	NI	St. Catharines	larva	43.11008334	-79.2645
INI90-3	*An. quadrimaculatus s.l.*	19-Sep-07	NI	St. Catharines	larva	43.11008334	-79.2645
INI90-4	*An. quadrimaculatus s.l.*	19-Sep-07	NI	St. Catharines	larva	43.11008334	-79.2645
INI91-6	*An. diluvialis*	19-Sep-07	NI	Pelham	larva	43.0975	-79.33141667
INI94-16	*An. smaragdinus*	8-Oct-07	NI	St. Catharines	adult	43.1954	-79.18525
INI64-11	*An. smaragdinus*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
INI64-12	*An. smaragdinus*	21-Aug-07	NI	Virgil	adult	43.1954	-79.18525
INI60-6	*An. smaragdinus*	17-Aug-07	NI	Pelham	adult	43.102	-79.30375
NI216-16-1	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.883	-79.2781
NI216-16-2	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.883	-79.2781
NI216-16-3	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.883	-79.2781
NI216-16-4	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.883	-79.2781
NI206-6-1	*An. quadrimaculatus s.l.*	30-Jul-09	NI	Wainfleet	larva	42.881	-79.313
NI206-6-2	*An. quadrimaculatus s.l.*	30-Jul-09	NI	Wainfleet	larva	42.881	-79.313
NI206-6-3	*An. quadrimaculatus s.l.*	30-Jul-09	NI	Wainfleet	larva	42.881	-79.313
NI206-6-4	*An. quadrimaculatus s.l.*	30-Jul-09	NI	Wainfleet	larva	42.881	-79.313
INO17-7	*An. earlei*	16-Jul-05	NO	Espanola	larva	46.18083	-81.71944
INO17-8	*An. earlei*	16-Jul-05	NO	Espanola	larva	46.18083	-81.71944
INO17-9	*An. earlei*	16-Jul-05	NO	Espanola	larva	46.18083	-81.71944
LP01-15	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
LP01-16	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
LP01-17	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
LP01-18	*An. walkeri*	26-Aug-08	LP	Long Point	female	42.5824	-80.3898
IBC57-30	*An. freeborni*	18-Aug-08	BC	Kamloops	female	50.852	120.2978
IBC57-31	*An. freeborni*	18-Aug-08	BC	Kamloops	female	50.852	120.2978
IBC57-32	*An. freeborni*	18-Aug-08	BC	Kamloops	female	50.852	120.2978
INI218-50	*An. barberi*	20-Aug-09	NI	Niagara Falls	female	43.0625	-79.1745
INI218-51	*An. barberi*	20-Aug-09	NI	Niagara Falls	female	43.0625	-79.1745
INI218-52	*An. barberi*	20-Aug-09	NI	Niagara Falls	female	43.0625	-79.1745
INI218-53	*An. barberi*	20-Aug-09	NI	Niagara Falls	male	43.0625	-79.1745
INI45-2	*An. quadrimaculatus s.l.*	25-Jul-06	NI	Port Colbourne	larva	42.92957	-79.25502
INI45-3	*An. quadrimaculatus s.l.*	25-Jul-06	NI	Port Colbourne	larva	42.92957	-79.25502
INI60-10	*An. quadrimaculatus s.l.*	17-Aug-07	NI	Pelham	larva	43.102	-79.30375
INI60-11	*An. quadrimaculatus s.l.*	17-Aug-07	NI	Pelham	larva	43.102	-79.30375
IWE35-5	*An. quadrimaculatus s.l.*	19-Sep-05	WE	Leamington	larva	42.03083	-82.51556
IWE35-6	*An. quadrimaculatus s.l.*	19-Sep-05	WE	Leamington	larva	42.03083	-82.51556
IWE35-7	*An. quadrimaculatus s.l.*	19-Sep-05	WE	Leamington	larva	42.03083	-82.51556
IWE26-1	*An. quadrimaculatus s.l.*	18-Sep-05	WE	Amherstburg	larva	42.03611	-83.03806
IWE26-2	*An. quadrimaculatus s.l.*	18-Sep-05	WE	Amherstburg	larva	42.03611	-83.03806
IWE31-3	*An. quadrimaculatus s.l.*	18-Sep-05	WE	Amherstburg	larva	42.18694	-83.08778
IWE31-4	*An. quadrimaculatus s.l.*	18-Sep-05	WE	Amherstburg	larva	42.18694	-83.08778
IWE29-10	*An. quadrimaculatus s.l.*	19-Sep-05	WE	Windsor	larva	42.33167	-82.92639
IOT23-2	*An. quadrimaculatus s.l.*	28-Aug-05	OT	Morton	larva	44.53917	-76.18472
INO12-10	*An. earlei*	15-Jul-05	NO	Manitoulin	larva	45.78667	-81.94111
IBC57-20	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
IBC57-21	*An. punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
INO12-11	*An. earlei*	15-Jul-05	NO	Manitoulin	larva	45.78667	-81.94111
INO12-12	*An. earlei*	15-Jul-05	NO	Manitoulin	larva	45.78667	-81.94111
IBC57-33	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
IBC57-34	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978

Appendix III (con't)

Code	Species	Date	Region	City	Stage	Latitude	Longitude
1BC57-35	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-36	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1NI225-18	*An. barberi*	27-Sep-09	NI	Niagara Falls	larva	43.0625	-79.1745
1NI225-19	*An. barberi*	27-Sep-09	NI	Niagara Falls	larva	43.0625	-79.1745
1NI225-20	*An. barberi*	27-Sep-09	NI	Niagara Falls	larva	43.0625	-79.1745
1NI225-21	*An. barberi*	27-Sep-09	NI	Niagara Falls	larva	43.0625	-79.1745
1NI225-22	*An. barberi*	27-Sep-09	NI	Niagara Falls	larva	43.0625	-79.1745
1NI225-23	*An. barberi*	27-Sep-09	NI	Niagara Falls	larva	43.0625	-79.1745
1NI217-2	*An. perplexens*	20-Aug-09	NI	Chippawa	larva	43.0227	-79.0885
1NI217-3	*An. perplexens*	20-Aug-09	NI	Chippawa	larva	43.0227	-79.0885
1NI217-4	*An. punctipennis*	20-Aug-09	NI	Chippawa	larva	43.0227	-79.0885
1NI215-6	*An. perplexens*	17-Aug-09	NI	Port Colbourne	larva	42.8831	-79.2781
1NI219-3	*An. perplexens*	29-Aug-09	NI	Flambourough	larva	43.3124	-80.0778
1NI222-7	*An. perplexens*	4-Sep-09	NI	Wainfleet	larva	42.8949	-79.2912
1BC57-38	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-39	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-40	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-41	*An. freeborni/punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-42	*An. freeborni/punctipennis*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-43	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-44	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-45	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC57-46	*An. freeborni*	18-Aug-08	BC	Kamloops	larva	50.852	120.2978
1BC58-6b	*An. punctipennis*	27-Jun-08	BC	Chilliwack	larva	49.2	-121.7333
1BC58-7b	*An. punctipennis*	27-Jun-08	BC	Chilliwack	larva	49.2	-121.7333
1BC58-8	*An. punctipennis*	27-Jun-08	BC	Chilliwack	larva	49.2	-121.7333
1NF2-18	*An. earlei*	7-Apr-06	NF	Deer Lake	adult	49.21623	-57.33337
1RQ11-1	*An. earlei*	18-Jun-06	RQ	Radisson	adult	53.66448333	-78.32241667
1RQ25-10	*An. earlei*	20-Jun-07	RQ	Radisson	adult	53.499	-77.45178333
1RQ24-2	*An. earlei*	20-Jun-07	RQ	Radisson	adult	53.52266667	-77.46783333
1RQ24-3	*An. earlei*	20-Jun-07	RQ	Radisson	adult	53.52266667	-77.46783333
1RQ24-4	*An. earlei*	20-Jun-07	RQ	Radisson	adult	53.52266667	-77.46783333
1NO4-1	*An. perplexens*	14-Jul-05	NO	Manitoulin	adult	45.7125	-82.2175
1NO4-2	*An. perplexens*	14-Jul-05	NO	Manitoulin	adult	45.7125	-82.2175
1NO4-4	*An. perplexens*	14-Jul-05	NO	Manitoulin	adult	45.7125	-82.2175
1NO9-2	*An. perplexens*	15-Jul-05	NO	Manitoulin	adult	45.76139	-82.15583
1NO4-3	*An. perplexens*	14-Jul-05	NO	Manitoulin	adult	45.7125	-82.2175
1HL3-4	*An. perplexens*	14-Jul-05	HL	Milton	adult	43.35778	-79.99444
1HL3-3	*An. perplexens*	14-Jul-05	HL	Milton	adult	43.35778	-79.99444
1AL24-1	*An. perplexens*	8-Feb-05	AL	Algonquin	adult	45.57167	-78.42944
1AL24-3	*An. perplexens*	8-Feb-05	AL	Algonquin	adult	45.57167	-78.42944
1NO3-6	*An. earlei*	14-Jul-05	NO	Manitoulin	adult	45.78056	-82.15111
1NO3-7	*An. earlei*	14-Jul-05	NO	Manitoulin	adult	45.78056	-82.15111
1WE42-3	*An. punctipennis*	30-May-06	WE	Morpeth	adult	42.28889	-81.85278
1WE44-4	*An. punctipennis*	6-Feb-06	WE	Leamington	adult	41.95027	-82.51728
1WE45-2	*An. punctipennis*	31-May-06	WE	Leamington	adult	41.96083	-82.52722
1WE45-3	*An. punctipennis*	31-May-06	WE	Leamington	adult	41.96083	-82.52722
1NO3-5	*An. perplexens*	14-Jul-05	NO	Manitoulin	adult	45.78056	-82.15111
1NO4-5	*An. perplexens*	14-Jul-05	NO	Manitoulin	adult	45.7125	-82.2175
1WE132-1	*An. punctipennis*	15-Jun-08	WE	Rondeau	larva	42.2762	-81.9678
1WE101-1	*An. punctipennis*	6-Dec-08	WE	Leamington	larva	41.9504	-82.5172
1WE104-1	*An. quadrimaculatus s.l.*	6-Dec-08	WE	Leamington	larva	42.0406	-82.5091
1WE110-1	*An. quadrimaculatus s.l.*	13-Jun-08	WE	Amherstburg	larva	43.0645	-82.0741
1WE132-2	*An. punctipennis*	15-Jun-08	WE	Rondeau	larva	42.2762	-81.9678

Appendix III (con't)

Code	Species	Date	Region	City	Stage	Latitude	Longitude
1WE103-1	*An. punctipennis*	14-Jul-05	WE	Leamington	larva	41.9499	-82.5164
1WE103-2	*An. quadrimaculatus s.l.*	14-Jul-05	WE	Leamington	larva	41.9499	-82.5164
1BP09-1	*An. quadrimaculatus s.l.*	20-Jun-08	BP	Bruce Peninsula	larva	45.2228	-81.4712
1BP09-2	*An. punctipennis*	20-Jun-08	BP	Bruce Peninsula	larva	45.2228	-81.4712
1BP14-1	*An. punctipennis?*	21-Jun-08	BP	Bruce Peninsula	larva	45.141	-81.4571
1BP19-1	*An. punctipennis*	22-Jun-08	BP	Tobermory	larva	45.263953	-81.7021
1NI222-1	*An. quadrimaculatus s.l.*	9-Apr-09	NI	Wainfleet	larva	42.8949	-79.2912
1NI222-2	*An. punctipennis*	9-Apr-09	NI	Wainfleet	larva	42.8949	-79.2912
1NI222-3	*An. perplexens*	9-Apr-09	NI	Wainfleet	larva	42.8949	-79.2912
1NI222-4	*An. punctipennis*	9-Apr-09	NI	Wainfleet	larva	42.8949	-79.2912
1NI222-5	*An. quadrimaculatus s.l.*	9-Apr-09	NI	Wainfleet	larva	42.8949	-79.2912
1NI222-6	*An. quadrimaculatus s.l.*	9-Apr-09	NI	Wainfleet	larva	42.8949	-79.2912
1NI226-5	*An. punctipennis*	27-Sep-09	NI	St. Catharines	larva	43.1095	-79.2646
1NI213-1	*An. punctipennis*	16-Aug-09	NI	Wainfleet	larva	42.8561	-79.3458
1NI213-2	*An. punctipennis*	16-Aug-09	NI	Wainfleet	larva	42.8561	-79.3458
1NI213-3	*An. quadrimaculatus s.l.*	16-Aug-09	NI	Wainfleet	larva	42.8561	-79.3458
1NI209-1	*An. punctipennis*	8-Jun-09	NI	Fort Erie	larva	43.0358	-79.0604
1NI209-2	*An. punctipennis*	8-Jun-09	NI	Fort Erie	larva	43.0358	-79.0604
1NI215-1	*An. quadrimaculatus s.l.*	17-Aug-09	NI	Port Colbourne	larva	42.8831	-79.2781
1NI215-2	*An. quadrimaculatus s.l.*	17-Aug-09	NI	Port Colbourne	larva	42.8831	-79.2781
1NI215-3	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.8831	-79.2781
1NI215-4	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.8831	-79.2781
1NI215-5	*An. punctipennis*	17-Aug-09	NI	Port Colbourne	larva	42.8831	-79.2781
1NI217-1	*An. perplexens*	20-Aug-09	NI	Chippawa	larva	43.0227	-79.0885
1NI217-2	*An. punctipennis*	20-Aug-09	NI	Chippawa	larva	43.0227	-79.0885
NI203-7-1	*An. perplexens*	7-Oct-09	NI	Pelham	larva	43.099745	-79.332093
NI203-7-2	*An. perplexens*	7-Oct-09	NI	Pelham	larva	43.099745	-79.332093