Revisionary systematics and evolutionary ecology of Neophylidorea (Diptera: Tipuloidea)

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Revisionary systematics and evolutionary ecology of Neophylidorea (Diptera: Tipuloidea)

by

Jessica Diane Petersen

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Entomology; Ecology and Evolutionary Biology

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Iowa State University
Ames, Iowa
2010

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DEDICATION

I dedicate this dissertation to my dear friend and colleague Dr. Ron VanNimwegen.
TABLE OF CONTENTS

ABSTRACT .............................................................................................................................................. VII

CHAPTER ONE: DISSERTATION OVERVIEW ...................................................................................... 1

Crane Flies ........................................................................................................................................... 3
Dissertation Organization ................................................................................................................... 5
References ........................................................................................................................................... 6

CHAPTER TWO: BOTTOM-UP SOLUTION TO THE CRANE FLY CONFUSION: DESCRIPTION OF
A NEW GENUS *NEOPHYLIDOREA* (DIPTERA: TIPULOIDEA) .................................................... 12

Abstract ............................................................................................................................................... 12
Introduction ........................................................................................................................................... 12
Classification of “Limnophilinae” .......................................................................................................... 13
Taxonomic History of Euphyliodorea ..................................................................................................... 15
Possible Solutions ................................................................................................................................. 16

Methods ................................................................................................................................................ 17
Taxonomy ............................................................................................................................................. 18
Discussion ............................................................................................................................................. 22
References ............................................................................................................................................. 24
Acknowledgements ................................................................................................................................. 27
Figures ................................................................................................................................................... 28
Tables ..................................................................................................................................................... 31

CHAPTER THREE: INTEGRATING MORPHOLOGY, MOLECULAR AND ECOLOGICAL DATA TO
DELIMIT SPECIES: SUPPORT FOR NEW COMBINATIONS OF *NEOPHYLIDOREA* (DIPTERA:
TIPULOIDEA) ......................................................................................................................................... 34
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>34</td>
</tr>
<tr>
<td>Keywords</td>
<td>35</td>
</tr>
<tr>
<td>Introduction</td>
<td>35</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>38</td>
</tr>
<tr>
<td>Morphospecies Hypotheses</td>
<td>38</td>
</tr>
<tr>
<td>Research Specimens</td>
<td>38</td>
</tr>
<tr>
<td>Morphological Methods</td>
<td>39</td>
</tr>
<tr>
<td>Geometric Morphometrics Methods</td>
<td>40</td>
</tr>
<tr>
<td>Molecular Sequencing</td>
<td>41</td>
</tr>
<tr>
<td>Molecular Delineation Methods</td>
<td>43</td>
</tr>
<tr>
<td>Ecological Niche Modeling Methods</td>
<td>44</td>
</tr>
<tr>
<td>Results</td>
<td>46</td>
</tr>
<tr>
<td>Morphological Results</td>
<td>46</td>
</tr>
<tr>
<td>Geometric Morphometrics Results</td>
<td>48</td>
</tr>
<tr>
<td>Molecular Sequence Results</td>
<td>48</td>
</tr>
<tr>
<td>Ecological Niche Identity Results</td>
<td>51</td>
</tr>
<tr>
<td>Discussion</td>
<td>52</td>
</tr>
<tr>
<td>Morphology</td>
<td>53</td>
</tr>
<tr>
<td>Geometric Morphometrics</td>
<td>55</td>
</tr>
<tr>
<td>Molecular Sequences</td>
<td>56</td>
</tr>
<tr>
<td>Niche Differentiation</td>
<td>57</td>
</tr>
<tr>
<td>Indiscrete adusta – columbiana group</td>
<td>59</td>
</tr>
<tr>
<td>Implications for Crane Fly Systematics</td>
<td>59</td>
</tr>
<tr>
<td>Limitations</td>
<td>60</td>
</tr>
<tr>
<td>Potential Mechanisms of Evolution</td>
<td>61</td>
</tr>
<tr>
<td>Conclusions</td>
<td>62</td>
</tr>
<tr>
<td>Funding</td>
<td>62</td>
</tr>
</tbody>
</table>
CHAPTER FOUR: SYSTEMATIC REVISION AND PHYLOGENETICS OF *NEOPHYLIDOREA*
(DIPTERA: TIPULOIDEA) ........................................................................................................... 102

Abstract ........................................................................................................................................ 102

Keywords ........................................................................................................................................ 102

Introduction ...................................................................................................................................... 102

  Taxonomic History ......................................................................................................................... 104

Materials and Methods .................................................................................................................... 105

  Taxonomy ..................................................................................................................................... 105

  Phylogenetics ................................................................................................................................. 108

Results ............................................................................................................................................. 110

  Taxonomy ..................................................................................................................................... 110

    *Neophylidorea caudifera* (Alexander, 1927) ............................................................................. 111

    *Neophylidorea flavapila* (Doane 1900) .................................................................................. 113

    *Neophylidorea vanronea*, sp. nov. ........................................................................................... 119

    *Neophylidorea neadusta* (Alexander 1927) .......................................................................... 120
Neophylidorea columbiana (Alexander 1927) ................................................................. 121
Neophylidorea adusta (Osten Sacken 1859) ................................................................. 127
Additional Material Examined .................................................................................... 134
Doubtful Species ........................................................................................................ 135
Taxonomic Key to the Adult Males of Neophylidorea .............................................. 135
Phylogenetics ............................................................................................................. 136
Discussion .................................................................................................................... 140
References .................................................................................................................. 142
Acknowledgements .................................................................................................... 148
List of Figures ............................................................................................................. 150
Figures ....................................................................................................................... 153
Tables ......................................................................................................................... 165

CHAPTER FIVE: GENERAL CONCLUSIONS ................................................................. 168
Direction of Future Research ..................................................................................... 170
References .................................................................................................................. 171

ACKNOWLEDGEMENTS ............................................................................................. 173
ABSTRACT

Uncovering the evolutionary and ecological process that has lead to biodiversity has been, and continues to be one of the most fascinating aspects of biology. One way to assess such processes is to study the diversity and limits of species. One group of interest in biodiversity are the crane flies (Diptera: Tipuloidea) because of their extreme diversity (>15,000 described species, representing 10% of all Diptera). Little is known about the patterns and processes responsible for creating such species diversity in crane flies. Often termed the fundamental units of life, species remain a difficult entity to define. Through recent innovations in analytical techniques and theoretical underpinnings, the process of delimiting species has resurged in interest. These new procedures have opened the door to define species like never before. Before defining the limits of species, a monophyletic group of species must be accurately described so as to include the most relevant species. I defined a new genus based on unique aspects of the male genitalia in a group of crane flies (Diptera: Tipuloidea). Based on key morphological features, 15 species were placed within the new genus, *Neophylidorea*. A preliminary revision of the group indicated that the diversity was perhaps over-estimated. I used four criteria and a variety of multivariate methods to delimit species of *Neophylidorea*. Based on the criteria used including morphology, geometric morphometrics, molecular sequences and ecological niche divergence, the four morphospecies built from the preliminary revision represent distinct, independently evolving lineages. A formal revision of these four species, and two additional species that lacked data sufficient to perform detailed analyses, concludes this dissertation. One of the two species lacking data is a newly described species to science.
CHAPTER ONE: DISSERTATION OVERVIEW

This dissertation follows a little known group of flies through a modern day taxonomic revision by first incorporating cladistic approaches to describing a new genus, then delimiting species using multiple, cutting-edge methods, and finally formally revising the clearly delimited species. The methods used were a holistic, integrated approach to studying evolution through morphological, molecular and ecological data to investigate the limits of species and their evolutionary histories.

A major undertaking of systematics is to discover and describe species, but this process is far from straightforward or simple. Species are the fundamental unit of biology and are used as such in analyses of biodiversity, population and community ecology, biogeography, and evolutionary biology (Riddle and Hafner 1999, Purvis and Hector 2000, Mayden 2002). Effects of delimiting species can have impacts in fields such as pest management (Ross and Shoemaker 2005), conservation biology (Agapow et al. 2004, Isaac et al. 2004, Johnson et al. 2004) and public health (Gentile et al. 2000).

Identifying species has long been a controversial undertaking (Dobzhansky 1935); even Darwin considered the idea to be problematic (Pigliucci 2003). Many researchers have since proposed their solution to the “species problem” (e.g., Ghiselin 1974, Ridley 1989, de Queiroz 2005), but clearly the problem has yet to be fully resolved. Reasons for the longstanding controversy mainly revolve around theoretical constructs of what a species is and what “concept(s)” or measures should be used to define a species (Mayden 1997). There are scores of alternative definitions for the term “species” (Coyne and Orr 2004) ranging from ecological, morphological, or phylogenetic in nature, just to name a few.

Recent arguments have been made to consider species concepts and methods for delimiting species in two separate constructs (Frost and Kluge 1994; Mayden 1997; Mayden 1999; Hey 2006b; de Queiroz 2007). By separating the theoretical concept of species (the primary concept) from the criteria used to define species limits (the secondary concept), the arguments surrounding which “concept” is best are somewhat removed from the situation. One common theme throughout many species concepts is the idea of identifying independent lineages. The metapopulation lineage concept suggests primarily approaching the species problem through this unified theoretical concept.
(de Queiroz 2007). Metapopulation lineages are identified as separate segments and independently evolving. The next step in the concept is to utilize any number of operational criteria to provide support for species or other levels of taxonomic structure. The metapopulation lineage concept is used throughout this dissertation to identify taxa at various hierarchical taxonomic levels. Various secondary criteria are used to identify species such as morphology, molecular sequences, and ecology.

Multiple aspects of biology have historically and contemporarily been included in various criteria used to delimit species. Morphology is essential to almost all aspects of biology. At the heart of every biologist’s research is an organism, each with some sort of unique morphology that may have been used to identify that particular organism. Therefore, linking the evolutionary history or relatedness of different organisms (species or individuals) to their respective morphologies is essential for understanding phenotypes and phenotypic change (Wiens 2004). Molecular data can help uncover the past in a way that continues to amaze (e.g., Hebert et al. 2004, Condon et al. 2008). Molecular systematics is a burgeoning field (Caterino et al. 2000), but much work needs to be completed before sequence data can be reliably used to delimit species. This is especially true for arthropods, where mitochondrial DNA does not always provide a reliable “barcode” (e.g., Whitworth et al. 2007, Virgilio et al. 2010), despite the need and desire for a rapid means of reliably delimiting species. Ecological data has much to offer in terms of identifying the potential mechanisms behind the formation of species (Coyne and Orr 2004). While a single method has yet to be developed that can identify species with 100% accuracy (except see Hebert et al. 2003), support from many methods must be employed and interpretations made with caution.

Researchers are confronted with a theoretical balancing act when these various forms of data do not tell the same story. This has historically resulted in conflict over which data tell the “correct” story and ongoing commentary on the differences among concepts (e.g., Dobzhansky 1935, Donoghue 1985, Baum and Donoghue 1995, Hey 2006a). As suggested by de Queiroz (2007) different criteria will identify lineage divergence at different points during speciation. Recently diverged taxa are the most complicated scenario when identifying independent lineages (Shaffer and
Thomson 2007). My argument throughout this dissertation is that multiple datasets should be garnered, considered, analyzed and interpreted for what they are and what they potentially represent. Only then can we understand where discrepancies lie and what factors may have lead to discordance.

There is a wealth of new data and analytical techniques (Wiens and Servedio 2000, Wiens and Penkrot 2002, Hey 2006a, Rissler and Apodaca 2007, Warren et al. 2008, Carstens and Dewey 2010, Leaché and Fujita 2010) to define species limits. These have only recently become available, each with different insight and assumptions of lineage divergence. These new analytical approaches, criteria and data have created what some have termed a “Renaissance issue” in systematic biology, focusing more efforts towards utilizing multiple datasets to delimit species (Sites and Marshall 2003, Sites and Marshall 2004).

Following the delimiting of species, it is important to proceed with a formal description or redescription of taxa (Simpson 1961). The detailed account of species morphology, phenology, geographic distribution and biology is important to fully disclose the limits of species. Along with a revision comes practical information such as a taxonomic key to species. All of these details are necessary to not only document the revised taxonomy, but to provide these resources that can be used by researchers attempting to identify species for purposes other than systematics.

**Crane Flies**

Crane flies (Diptera: Tipuloidea) are the most diverse group of flies (>15,000 species, 10% of all Diptera; de Jong et al. 2008, Oosterbroek 2010), but the family suffers from a taxonomic impediment and lack of biological information. Many species are ill-defined, lacking fundamental systematic information such as phylogenetic hypotheses, adequate species descriptions, taxonomic keys and basic life history information (e.g., only 4% of adults have immature associations). Crane flies are ecologically influential to community dynamics (e.g., in Pennsylvania, crane flies represent 2% of the state-wide biodiversity, Young & Gelhaus 2000) and ecosystem processing as indicated by their relative abundance in spring habitats (Gathmann and Williams 2006). But unless there is a solid
taxonomic foundation including identification keys, examples of DNA association of life stages, and basic life history data, more applied research cannot progress.

One subfamily has particularly problematic taxonomy, the “Limnophilinae”. This group in particular is large (approximately 2300 recognized species, Oosterbroek 2010) and includes many genera that are monotypic, many with over 100 species (e.g., *Hexatoma* Latreille, *Limnophila* Macquart) and many that need revisions. Two authors have recently identified “Limnophilinae” as paraphyletic (Ribeiro 2008, Petersen et al. 2010). The “Limnophilinae” need much more phylogenetic work at both higher (genus and above) and lower (species) levels. Aside from large overview of the group (Starý 1971), no cladistic revisions of “Limnophilinae” taxa exist. Some work has been done describing new species (Savchenko 1976), and new subgenera (Savchenko 1979) redescribing previously described taxa (Savchenko 1978, Ribeiro 2007) and describing aspects of “Limnophilinae” species distributions (Savchenko 1973), but these examples are few. Revisions are essential to forming an accurate representation of the diversity, providing resources for other researchers such as taxonomic keys, and an overall understanding of the phylogenetic relationships among taxa. From here we can ask interesting and insightful questions about, for example character evolution, community ecology and evolution, modes of speciation, effects of climate change, mechanisms of local diversity, and invasive species biology.

As an example, the genus *Euphyllidorea* Alexander was described particularly poorly (Alexander 1972). The genus was described late in the career of Charles Alexander, the most prolific worker in the field of crane fly taxonomy (Oosterbroek 2009). Prior to my work, this genus consisted of 51 species and one subspecies. Alexander’s work became more detailed with time, but even the most recent revisions were not necessarily based on a solid taxonomic framework. The genus *Euphyllidorea* was described based on a trifid aedeagus and a “short” Radial sector vein. Alexander goes on to describe the type species, *E. niveitarsis* Osten Sacken in greater detail. At the beginning of my research into *Euphyllidorea*, I surveyed all species within the genus to understand the morphology and potential synapomorphies pertaining to the group. Clearly the trifid aedeagus in *E. niveitarsis* was not necessarily homologous to the trifid aedeagus of other species (Alexander often
referred to one as the “adusta” or long trifid group, another as the “similis” or short trifid group; Alexander 1942). Additionally, many species of Euphyllidorea simply did not bear a trifid aedeagus, but rather had a single opening of the male intermittent organ. Because a complete revision of “Limnophilinae” (the paraphyletic subfamily in which these species are classified) would have been required to fully revise Euphyllidorea, and its monophyly was in question, I removed a subset of Euphyllidorea species that have multiple group-defining characters and redefined them as a new genus, Neophylidorea. The majority of the species I define as Neophylidorea (and two additional species that he overlooked) were identified by Alexander as the “adusta” group (1972).

Dissertation Organization

This dissertation is organized into five chapters. Chapter one is an overview of the general components of my dissertation, the underlying philosophy and the motivation for this research. Chapter two is a manuscript describing the new genus, Neophylidorea and a new species, N. vanronea. This was submitted to Invertebrate Systematics in April, 2010 and reviews were recently returned. Some corrections from the reviews have been incorporated into the current version of this manuscript. The basis for the remainder of the dissertation is developed in this chapter defining a monophyletic taxon. Chapter three builds upon the description of a new genus by using four operational criteria to delimit species of Neophylidorea. The four methods are discussed in detail and all generally agree with the morphological species hypothesis upon which each method is tested. The most laborious method used involved molecular analysis that was completed at the Cornell Lab of Ornithology in the Fuller Evolutionary Biology Lab, under the direction of Dr. Irby Lovette. As a visiting researcher in the Fuller Lab, I learned the techniques necessary to complete this aspect of my dissertation. Because of his level of involvement, Irby Lovette is a co-author on this paper to be submitted to Systematic Biology. Chapter four is a systematic revision of Neophylidorea describing the morphological limits and biology of each species. Included here is also a species-level phylogenetic hypothesis and taxonomic key to the species of Neophylidorea. This final research chapter will be submitted to Systematic Entomology. Each of the three research chapters are
formatted for the respective target journals with myself as the primary author. I am the primary researcher and author of all three main chapters. The ultimate chapter is a general overview, synthesizing all that was gained from this dissertation and potential future directions for this work.

The descriptions of new taxa contained herein are the basis for descriptions in separate published works (i.e., refereed journals); under Article 8.2 of the International Code of Zoological Nomenclature, the author requests that neither this thesis, nor the descriptions contained herein, be considered “published” within the meaning of the code (ICZN 2000).

References


CHAPTER TWO: BOTTOM-UP SOLUTION TO THE CRANE FLY CONFUSION: DESCRIPTION OF A NEW GENUS NEOPHYLIDOREA (DIPTERA: TIPULOIDEA)

Running head: New crane fly genus Neophylidorea

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Abstract

Crane fly systematics is wrought with difficulties at multiple hierarchical levels. The current taxonomy lacks evolutionary structure at the generic level for many groups, which may be causing incongruent phylogenetic hypotheses at the subfamilial and familial levels. Here we restructured species to describe a monophyletic genus that will in part help pave the way for more robust higher level phylogenetics. A group of 16 species is removed from the existing genus, Euphylidorea Alexander based on distinct morphology and described as the new genus Neophylidorea. Characters of the aedeagus and male genitalia in general allow for unequivocal recognition of this genus. There are few consistent differences in macroscopic or female morphology between species of Neophylidorea gen.nov.

Keywords: “Limnophilinae”, Tipuloidea, Euphylidorea

Introduction

Crane flies (Diptera: Tipuloidea) are the most taxonomically diverse superfamily of flies (>15,000 species, 10% of all Diptera; Oosterbroek 2010). Although taxonomically diverse, little modern revisionary taxonomy of the Tipuloidea has been completed. This is especially true for the “Limoniidae” where many taxa are poorly defined, lacking fundamental systematic information such as phylogenetic hypotheses, taxonomic keys and life history information (Pritchard 1983). The paucity of basic biological information may have profound effects on ecological studies where, without the ability to identify species, research cannot progress despite a potentially large role for this group...
in ecosystem processing (Gathmann and Williams 2006). One goal of modern cladistics is to describe or redefine taxa such that they represent monophyletic evolutionary lineages. This description of a new genus of crane fly provides an example of one method of redefining taxonomic groups to represent evolutionary lineages (de Queiroz & Gauthier 1994).

Among practicing taxonomists there is disagreement with regards to classification and taxonomic ranks of crane fly groups. Although we will not reiterate what has been described in detail elsewhere (Ribeiro 2008, Petersen et al. 2010), the classification used throughout this manuscript follows that of the most recent phylogenetic analysis of the group (Petersen et al. 2010), which recognizes the crane flies, Tipuloidea, as a superfamily containing two families, Tipulidae and Pediciidae, and various subfamilies within. Regardless of semantics, the crane flies are an extremely diverse and complex group of insects. Charles P. Alexander was an avid worker, who wrote over 1000 publications and described over 10,000 species of Tipuloidea in his lifetime (Oosterbroek 2010), including 14 of the 16 species of Neophylidorea **gen. nov.** (Table 1). From his discussions, he clearly doubted aspects of his hypothesized taxonomy (Alexander 1942). With such an amazing body of work completed in one lifetime, it is not surprising that revisions would be necessary and additional hypotheses would be proposed. Few comprehensive revisions of crane fly groups have been completed (e.g., Byers 1961, Brodo 1987, Young 1987, Gelhaus 2005, Petersen 2008), relative to the total number of described species. The time has come for other researchers to investigate the taxonomy proposed by Alexander and others. Here, we outline a method for reconstructing taxonomic structure in taxa with undefined or ambiguous morphological limits to form clear, evolutionary significant groups.

**Classification of “Limnophilinae”**

The “Limnophilinae” are represented by 52 genera and 44 subgenera (Oosterbroek 2010). “Limnophilinae” are exceptionally poorly understood and classified (Ribeiro 2008). All group-defining characters for “Limnophilinae” are plesiomorphic (Ribeiro 2008). Of the four quantitative phylogenetic analyses, none have identified “Limnophilinae” as a monophyletic lineage and, when similar terminal

Confusion at the subfamilial level may be rooted in the taxonomic problems that exist within “Limnophilinae” genera. Many genera are non-monophyletic, including large genera such as Limnophila, Gynoplistia Westwood, and Hexatoma (Ribeiro 2008). Even Alexander, who described most of these taxa, stated that “the various subgenera of Limnophila are highly artificial and are chiefly maintained for convenience only. The exact definition of the limits of the subgenera Prionolabis, Phylidorea, and Limnophila s.s., has proved especially difficult” (Alexander 1942). Subfamilial phylogenetics may be impossible to resolve with the current taxonomic framework, especially when using species as the terminal taxa and then making inferences to the generic level. If the genera themselves do not represent independent evolutionary lineages, then extrapolations cannot be made.
**Taxonomic History of Euphylidorea**

The genus *Euphylidorea* suffers from taxonomic confusion, best described by Alexander referring to *Limnophila* (*Phylidorea*) as "a large number of species that are very difficult of exact definition" (1942). What is considered here as the current classification of the genus *Euphylidorea* is taken from the Catalogue of the Craneflies of the World (Oosterbroek 2010, Table 1). However, different authors refer to this same collection of species using various terminology: *Euphylidorea* (Oosterbroek 2010), *Limnophila* (*Euphylidorea*) (Alexander 1972), or *Phylidorea* (*Euphylidorea*) (Savchenko 1986, Podenas et al. 2006).

Most species of *Euphylidorea* were originally described as either *Limnophila* or *Phylidorea*. These initial descriptions and groupings were vague, lacking specific group-defining characters, and essentially described what is today considered "Limnophilinae". Species of *Euphylidorea* described prior to 1921 were originally described as *Limnophila*. In 1919, Alexander designated a handful of Nearctic species (*N. adusta*, *E. costata*, *E. fulvocostalis*, *E. insularis*, *E. lutea*, *E. novaeangliae*, *E. smilis*, and *E. terraenovae*) as *Limnophila* (*Phylidorea*), but did not give a diagnosis of the subgenus. Species described from 1924 to present were described as *Limnophila* (*Phylidorea*). In 1938, Edwards described the subgenus *Limnophila* (*Phylidorea*) and gave an account of the Palearctic species, many of which are today considered *Euphylidorea* (*E. aperta*, *E. dispar*, *E. fulvonervosa*, *E. lineola*, *E. meigeni*, *E. phaeostigma*), but included other species in the diagnosis that are considered *Phylidorea* (*P. abdominalis* (Staeger), *P. ferruginea* (Meigen), *P. glabricula* (Meigen), and *P. squalens* (Zetterstedt)). Alexander (1972) treated the subgenus *Limnophila* (*Euphylidorea*) as a distinct group of mostly Nearctic species that were previously designated as *Limnophila* (*Phylidorea*).

Oosterbroek’s catalogue (2010) considers *Euphylidorea* a genus with a clarifying note that Alexander considered *Euphylidorea* to be a subgenus of *Limnophila*. Some species considered under the subgenus *Phylidorea* prior to Alexander’s description of *Euphylidorea* in 1972 were not listed by Alexander in that description (*N. columbiana*, *E. fuscovenosa*, *E. microphallus*, *E. platyphallus*, *E. subadusta*, *E. subsimilis*), or were listed as *Limnophila* (*Phylidorea*) (*E. fratia* and *E. lutea* but are considered as *Euphylidorea* by Oosterbroek (2010) without a published basis. One species, *E. 
O. insularis was placed as *Euphylidorea* by Woodley and Hilburn (1994). Regardless of the source of information, the taxonomy of *Euphylidorea* is unclear.

Not only is the taxonomy difficult to understand, but the morphological limits of *Euphylidorea* are not explicitly stated. A clear morphological description of *Euphylidorea* is lacking in the original description, which according to Alexander (1972) differs from other *Phylidorea* based on a trifid aedeagus. Savchenko (1986) indicated the difference between female specimens of these groups was a “short” Radial sector wing vein in *Euphylidorea*. Morphological differences between Palearctic species of *Phylidorea* and *Euphylidorea* are that of a simple versus trifid aedeagus (Podenas et al. 2006). This does not hold true for Nearctic species because not all species currently considered under the classification of *Euphylidorea* have a trifid aedeagus (e.g., *E. lutea*). Whether or not an aedeagus is “trifid” seems to be a matter of interpretation. For example, Ribeiro (2008) scores *E. niveitarsis* as having a simple aedeagus (character 87), despite the original description and sketches made by Alexander clearly indicating a trifid apex to the aedeagus. Some of the confusion may be explained by identifying a difference between the aedeagal filaments and the sheath surrounding those filaments, but this does not necessarily resolve the discrepancy between the original description and those *Euphylidorea* species that bear a singular aedeagus.

**Possible Solutions**

Clearly both the taxonomic structure and morphological limits of *Euphylidorea* are rather elusive. Two potential avenues for resolving this confusion exist. One method of tackling taxonomic problems is a top-down approach of resolving the classification based on representative species. A second method, employed here, involves redescribing taxa from the bottom-up to represent distinct evolutionary lineages.

The top-down approach was recently assessed by Ribeiro (2008) in his phylogenetic analysis of “Limnophilinae”. Although important to understanding higher-level evolutionary relationships and resolving taxonomic problems such as identifying paraphyletic genera, this approach is perhaps premature for “Limnophilinae” given the current lack of resolution at lower taxonomic levels.
Problems with the top-down approach for “Limnophilinae” include: 1. Unclear as to which species are representative if genera lack evolutionary structure, 2. Difficult to choose characters representative of a poorly understood genus. Including multiple species from particularly large genera may help resolve a portion of the taxonomic problems in the crane flies, but this top-down approach does not take into consideration genera lacking exact definitions.

The objective of this research is to clearly describe a new genus of “Limnophilinae” from a group of species formerly described as *Euphylidorea*. A bottom-up approach is used to resolve a portion of the “Limnophilinae” taxa by describing a new genus, *Neophylidorea*, including a new species and providing directions for future researchers.

**Methods**

Over 1000 specimens from various museums (Table 2) and personal collections were studied to understand the morphology of *Euphylidorea* sensu lato and *Phylidorea*. Most museum specimens were pinned or pointed, with male genitalia often preserved in glycerin in a microvial or slide mounted in Canada balsam. Additional specimens were collected during trips in 2007, 2008 and 2009 to study the biology, better understand the geographic distribution and collect fresh specimens. Field collected specimens were swept from vegetation and direct killed into 95% ethyl alcohol (EtOH), although some specimens were collected into ethyl acetate, stored in glassine envelopes and pointed as vouchers. Specimens were deposited at ISIC and USNM (Table II).

Rearing of larvae was attempted from eggs obtained from a female by using methods similar to those described by Young (2009). Eggs were reared to first-instar larvae while traveling on a collecting trip in 2009 using moist filter paper and commercial fish food. This did not prove to be especially successful, but better laboratory conditions beyond those available while traveling may have assisted.

The characters and terminology used in these descriptions follow McAlpine (1981) and Alexander and Byers (1981). Terminology adopted for wing veins is indicated in Figure 1A.
Taxonomy

Family TIPULIDAE
Subfamily “LIMNOPHILINAE”
Genus Neophylidorea, gen. nov.

Figs 1-3

Type-species: Limnophila adusta Osten Sacken, 1859: 235, original designation.

Diagnosis. Distinguished from other “Limnophilinae” by the presence of three filaments of the aedeagus, each of which extends at least three times longer than the common stem. The median aedeagal filament curves dorsally away from the lateral filaments just apical to the split from the short common stem. Additional diagnostic morphological features include median process on the ninth tergite of variable shape, outer gonostylus hooked apically in a finger-like projection, aedeagal filaments entire, and relatively short Rs vein.

Description. Larva (Fig 1B). Hemicephalic, maxillae divergent, curved, tusk-like, with apices visible when head is withdrawn, mandibles sickle shaped. Densely, matted, brown setae cover larval surface. Large bulbous structure anterior to spiracular disk, as typical of “Limnophilinae”. Spiracular disk with four lobes; inner dorsal surface of ventral lobes sclerotized, additional sclerotized surface and patch of setae near base, rounded apically; inner ventral surface of dorsal lobes sclerotized, apices rounded dorsally, forming a point ventrally; ventral lobes slightly longer than dorsal; thin fringe of yellow hairs surrounding each lobe, hairs longest at lobe tips, gradually diminishing in size; two spiracles, one on each dorsal lobe. Anal papillae with four pale, fleshy lobes.

Pupa. Undescribed.

Adult. A moderately sized yellow tipuline fly occasionally bearing darkening of the wing, thorax and terminal segments of the abdomen; male (N=119) body length 9.7mm (7.0-12.5mm), wing length 9.9mm (8.0-12.0mm); female (N=67) body length 10.5mm (6.5-14.0mm), wing length 10.3mm (7.5-13mm).

MALE. Head. Gray to black (appearing more black if preserved in EtOH), pruinose. Rostrum yellow to brown, palpi brown to black. Antenna. Generally short, scape brown to black, cylindrical
and elongate, sparsely pruinose; pedicel small, globular; flagellum 14-segmented as in "Limnophilinae", yellow to brown, 4-6 verticils per flagellomere, verticils on flagellomeres 1-2 shorter than the segment lengths, verticils on flagellomeres 3-4X longer than the segment, 1st flagellomere slightly distended, succeeding flagellomeres progressively more tubular with margins nearly straight, 1st flagellomere ca. 2X longer than wide, succeeding flagellomeres progressively becoming slightly shorter and thinner. Thorax. Lateral sclerites glabrous, yellow to brown; prescutum with two longitudinal rows of setae, often darker medially; setae on pronotum angled apically. Legs. Trochanter yellow, setae present; femora yellow apically with varying degree of darkening towards coxae; tibial spurs present, tarsal claws simple; tarsi dark brown to black. Wings (Fig 1A). Macrotretrichia on wing veins short and sparse, wing membrane without macrotretrichia. Yellow with varying degrees of darkening in specific regions within species: cord, along Rs, along CuA and apically. Oval stigma often prominent. Wing venation: arculus present, costal vein interrupted between R₄ and R₅, base of vein Rs variably spurred within species, Rs shape varies within species from curved to sharply angled, 3 branches of R and 3 branches of M reaching the wing margin, m-cu at ca. mid-point of the discal cell, M₁₂ ca. equal in length to M₁ or M₂, apex of the vein Sc reaches wing margin equal to or apical to the branch of Rs, Sc₂ distal to the origin of Rs, R₂ more or less perpendicular to the wing longitudinal axis, R₂ joins R₃ at ca. midpoint of R₃, R₄ parallel to R₅. Halter. Stem yellow with setae, knob infuscated. Abdomen: yellow to brown, often darker brown or black on segments 7, 8 and/or 9, sometimes with faint lateral and/or medial darkening (variable within species), sparsely pruinose. Genitalia (Fig 2A-F): 9th tergite membranously joined laterally to gonocoxites, posterior margin of the 9th tergite forms extensions (median lobe) that are either longer than wide (N. caudifera) or of equal width to length (all other species), usually pubescent; dorsal process present lateral to median lobe. Divided gonostylus situated terminally on the gonocoxite, inner gonostylus pubescent, angled at approximately mid-length with degree of the inner angle variable within species from 60 to 90 degrees; outer gonostylus hooked apically, glabrous, posterior margin smooth. Ejaculatory apodeme wide compared to width of the aedeagus pump, dorsoventrally flattened. Aedeagus split into three subequal filaments at the base, filaments curve dorsally then
straighten ending nearly equal to apical end of gonocoxite, all three filament sheath tips fluted to varying degrees (variation depends on method of preservation with slide mounting sometimes splitting these seams), all filaments entire from base to apex, medial sheath often slightly wider than two lateral sheaths with tip either pointed or bent at 90 degrees, aedeagal sheaths with varying degrees of sclerotization. Ventral parameres long, either bifid apically (*N. tepida, N. aleutica, N. flavapila*) or simple (all remaining species), and with varying degrees of sclerotization across species. Interbase bladelike, lacks sclerotization, apical shape variable between species, individualized from but articulated with dorsal parameres dorsally and gonocoxite apically, membranously joined medially.

**FEMALE (Figs 1C, 1D).** Similar to male except slightly larger as noted above. *Genitalia:* yellow to brown, three spherical and sclerotized spermathecae; cerci elongate, yellow, curved dorsally at apex; vaginal apodeme sclerotized, Y-shaped.

**Etymology.** The majority of the species considered here as *Neophylidorea* were originally described as *Phylidorea*. This new genus is named to reflect the historical taxonomy and also to help pave the way for a new era in Tipuloidea revisionary taxonomy.

**Geographic Distribution.** *Neophylidorea* is endemic to temperate cool regions of the Nearctic (Fig 3). Many species inhabit mountainous areas of western North America (*N. aequiatra, N. aleutica, N. brevifilosa, N. columbiana, N. flavapila, N. nevadensis, N. olympica, N. pacalis, N. snoqualmiensis,* and *N. tepida*) while other species are distributed throughout northeastern North America (*N. adusta, N. caudifera, N. neadusta,* and *N. paeneadusta*)

**Phenology.** Adults of *Neophylidorea* can be found as early as May 1, and as late as October 4. The highest likelihood of collecting adults of *Neophylidorea* is between June 1 and July 10. Larvae have been collected on May 16 (*N. adusta*) and June 27 (*N. flavapila*).

**Biology.** No detailed studies of the biology of any species of Neophylidorea have been conducted. In various papers, Alexander briefly commented on the habitat and the dominant plant community in the vicinity of his collecting. The following account is by no means complete and should be viewed as preliminary, anecdotal information.
Larvae inhabited wet, boggy areas such as fens, bogs, riparian areas and lake margins. Adults were found at the margins of these habitats, often in forested areas or open habitat near forest margin, on or near the ground or hanging from trees or shrubs. Species with western distributions (see ‘Geographic Distributions’) tended to inhabit open habitats such as wet meadows and bogs, whereas species with eastern distributions were collected from forested swamps and cedar-hemlock forests. Adults were easily collected by hand during early morning and late evening, when the air temperature was cool (13-20°C). Adults were collected near water sources that had surface water temperatures ranging from 8-13°C and pH of 5.5-6.8. Adults were usually collected along with other crane flies (e.g., Dolichopeza Curtis, Epiphragma, Limnophila, Molophilus Curtis, Pedicia Latreille and Tipula L.) and related flies (Bittacomorpha clavipes Fabr. and Ptychoptera Meigen).

In 2009, 12 of the 29 adult populations of N. adusta collected had a fungus that ranged from exteriorly encircling the terminal segments of the abdomen to almost entirely engulfing the lateral portions of the abdomen in addition to the terminus. Populations are relatively easily defined in Neophylidorea as a group of individuals inhabiting a particular closed habitat surrounded by uninhabitable environmental conditions. The fungus was identified as Entomophthorales, species of which have been noted in other Tipuloidea (Kramer 1980, Hajek et al. 2003). No other species of Neophylidorea collected by the authors had evidence of fungal infection. Individuals with the fungus exhibited physically hampered flight and were often collected on the ground. Females affected by the fungus did not contain eggs. Many infected individuals actually lacked terminalia or the last two to three segments became dismembered upon collection. During the same time, some other genera exhibited a similar fungal infection (Phylidorea, Tipula), but not all genera collected (e.g., Epiphragma, Limnophila, Pedicia (Tricyphona)).

Much intraspecific variation in the coloration of the abdomen and thorax appears to be associated with altitude, with higher altitude populations exhibiting darker pigmentation along the thorax.
Larvae were found to be predaceous, as expected given the known biologies of other "Limnophilinae" taxa. A single larva collected near Crane Prairie along Snow Creek, Oregon on June 27, 2007 contained nearly whole Chironomidae and Ceratopogonidae larvae in its digestive tract.

Remarks. Much of the macroscopic morphology is either consistent between species or variable within species. In the latter situations, the characters used to delimit species are microscopic and generally pertain to male genitalia. Additional research on delimiting species of Neophylidorea and redescribing species will be forthcoming.

Why Alexander (1972) listed N. caudifera and N. brevifilosa as bearing a "short" aedeagus, along with other "similis" species is unclear. In the original description he refers to N. caudifera as allied to "adusta", which he clearly indicates as bearing a long trifid aedeagus in various publications (Alexander 1942; 1972). The same is true for N. columbiana which was omitted from his list of species following the description of Euphylidorea. These three species were clearly mistakenly described as Euphylidorea and the various groups within.

Larval morphology was described from a single specimen collected from Oregon as noted above. Further sampling and identifications will likely improve upon this description. The larva was associated with an adult collected at the same locality through molecular techniques, which will be described in a forthcoming paper on delimiting species of Neophylidorea.

No pupae were collected during sampling trips, therefore a description is not included here. Alexander (1919, pg. 867) described what he supposed was the pupa of N. adusta, but the specimen was not reared to adult. Whether his description of the pupa and larva represent Euphylidorea and/or Neophylidorea gen. nov remains unclear. Rather than continue this line of reasoning by describing what can only be surmised from various pupal cases, a description of the pupa must await definitive association with the adult.

Discussion

Here, we described a new genus, Neophylidorea based on unique features of the male genitalia. This research provides a solid foundation upon which higher-level phylogenetics can be
robustly built. Like *Euphylidorea*, many crane fly genera are in need of revisionary systematics. This method of removing species from a genus lacking defined limits may prove useful for providing cladistic structure to crane fly genera. This method leaves in its wake, *Euphylidorea* as continued problematic taxa. We argue that placement of the remaining *Euphylidorea* taxa will continue to be problematic without a better understanding of higher-level phylogenetics. Although this approach is somewhat circular, both avenues to providing evolutionary structure to crane fly systematics have their place.

The objective of this research was not to reconstruct the phylogenetics of “Limnophilinae”, but rather to assess the taxonomic structure from the bottom-up. Some remarks regarding the potential placement of *Neophylidorea* can be made based on various Tipuloidea phylogenetic hypotheses. *Neophylidorea* scores identical to *Euphylidorea similis* in the analysis by Petersen et al. 2010. Because their analysis includes both sequence data and morphological data, the exact placement of *Neophylidorea* is unclear. Placement within the hypothesized phylogeny by Ribeiro (2008) is equally difficult for a variety of reasons.

First, Ribeiro considers *E. niveitarsis* as the representative species for *Euphylidorea*, but many of the character states are not equivalent in *Neophylidorea*. Bearing a trifid aedeagus is clearly an autapomorphic character within the “Limnophilinae” (Ribeiro 2008), which therefore underscores the monophyly of *Neophylidorea*. Another character is the trajectory of the aedeagus from a lateral perspective from the base to the apex (char. 67[1]). In all species of *Neophylidorea*, the aedeagus is clearly basally curved while in many species of *Euphylidorea*, including *E. niveitarsis* and those similar to *E. similis*, the aedeagus is straight. The ejaculatory apodeme of *Neophylidorea* is wide compared to the sperm pump and aedeagus (char. 70[1]) whereas in *E. niveitarsis* it is of subequal width to the aedeagus. The ventral parameres of *Neophylidorea* are variable between species with regard to character 72: simple and bifid. In those species similar to *E. similis*, the ventral paramere is bifid, while *E. niveitarsis* and potentially closely related species (i.e., *E. albipes*, *E. cherokensis*, and *E. globulifera*) have a simple ventral paramere. The ventral gonostylus is not sclerotized and simple in *Neophylidorea* (chars. 60[0] and 62[0]) while in *E. niveitarsis* it is weakly sclerotized and apically
bifid. The medial fusion of the interbase is another character that differs between groups, with *E. niveitarsis* having a fused interbase (char. 78[0]) and species close to *E. similis* and *Neophylidorea* having an interbase that is separated [1] medially. Lastly, the median lobe is an important character phylogenetically and is present in *Neophylidorea* (char. 52[1,2]), sometimes longer than wide (*N. caudifera*), but in most species wider than long. The *E. similis* group and *E. niveitarsis* do not have medial extensions on the ninth tergite [0], unlike *Neophylidorea* which have fleshy lobes on the ninth tergite.

The second reason for difficult placement of *Neophylidorea* within the Ribeiro phylogeny is that we do not agree with some of the scoring for *E. niveitarsis*. Ribeiro scores *E. niveitarsis* as bearing a simple aedeagus (char. 87[0]) whereas we would score both *E. niveitarsis* and *Neophylidorea* as trifid [1]. This conflicting interpretation, combined with the high degree of homoplasy in the dataset, does not allow inclusion of *Neophylidorea* in the matrix, and confounds any subsequent phylogenetic reconstruction. However, despite our disagreement with Ribeiro’s assessment of the number of terminal openings of the *E. niveitarsis* aedeagus, his analysis illuminates the uniqueness of this morphological feature.

It should be noted that the Ribeiro (2008) appendix mistakenly described the *E. niveitarsis* specimen localities as being from India. Following communication with Ribeiro, these three specimens of *E. niveitarsis* should read: *Euphylidorea niveitarsis* (Osten Sacken, 1859). *Limnophila niveitarsis* O.S., Det. C. P. Alexander, 1926 / Sport Island, Sacandaga R., NY. Alexander. [USNM] (and two additional specimens with the same information except both determined in 1925). Therefore, this was not the source of confusion surrounding our interpretation, as well as Alexander’s (1972) of *niveitarsis* as bearing a trifid aedeagus compared with that of Ribeiro (2008).

**References**


**Acknowledgements**

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Figure 1. *Neophylidorea adusta* wing (A), *N. flavapila* larval spiracular discs (B), *N. adusta* female vaginal apodeme, ventral view (C), and lateral terminalia view of *N. flavapila* in copula, male on left, female on right (D). Abbreviations: 8s = eight sternite, 9s = ninth sternite, 9t = ninth tergite, 10t = tenth tergite, A = anal veins, aed = aedeagus, C = costa, cd = common spermathecal duct, ce = cerci, CuA = anterior cubitus, dm = discal medial, hy = hypogynial valves, M = media, ml = median lobe, R = radius, Rs = radial sector, Sc = subcosta, sth = spermatheca.
Figure 2. *Neophylioidorea adusta* male hypopygium, dorsal view (A), *N. adusta* aedeagal complex, dorsal view (B), *N. adusta* male hypopygium, ventral view (D), *N. adusta* aedeagal complex, ventral view (D), *N. adusta* male hypopygium lateral view (E). Abbreviations: 9s = ninth sternite, 9t = ninth tergite, aed = aedeagus, d gonst = dorsal gonostylus, dpa = dorsal paramere, dpr = dorsal process, ej ap = ejaculatory apodeme, goncx = gonocoxite, ib = interbase, ml = median lobe, sp = sperm pump, v gonst = ventral gonostylus, vpa = ventral paramere.
Figure 3. Distribution of *Neophydidorea gen. nov.* (N=177).
Table I. Species considered *Neophylidorea* in this new genus description. Placement of *Euphylidorea* sensu lato taxa by various authors, Alexander and Oosterbroek. Museum holdings of holotypes where known are indicated. Our final determination of which taxa are considered *Neophylidorea* gen. nov. are listed in the last column.

<table>
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<td>modified</td>
<td>Euphytidorea</td>
<td>USNM</td>
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<tr>
<td><em>E.</em> subadusta (Alexander, 1924)</td>
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<tr>
<td><em>E.</em> subsimilis (Alexander, 1927)</td>
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<tr>
<td><em>N.</em> tepida (Alexander, 1926)</td>
<td>3 long</td>
<td>Euphytidorea</td>
<td>USNM</td>
<td>X</td>
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<td>modified</td>
<td>Euphytidorea</td>
<td>MCZ</td>
<td>X (female)</td>
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* Authors did not study these types, but the current location is indicated here.
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<th>Acronym</th>
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</tr>
<tr>
<td>CAS</td>
<td>California Academy of Sciences, San Francisco, California (N. Penny)</td>
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<td>CUIC</td>
<td>Cornell University Insect Collection (J. Liebherr)</td>
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<tr>
<td>FB</td>
<td>Personal collection of Fenja Brodo, Ottawa, Canada</td>
</tr>
<tr>
<td>ISIC</td>
<td>Iowa State Insect Collection, Ames, Iowa</td>
</tr>
<tr>
<td>MCZ</td>
<td>Museum of Comparative Zoology, Cambridge, Massachusetts (P. Perkins)</td>
</tr>
<tr>
<td>UMMZ</td>
<td>University of Michigan Museum of Zoology, Ann Arbor, Michigan (M.F. O'Brien)</td>
</tr>
<tr>
<td>USNM</td>
<td>Collections of the United States National Museum, deposited in the National Museum of Natural History (NMNH), Smithsonian Institution, Washington, D.C. (W.N. Mathis)</td>
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</table>
CHAPTER THREE: INTEGRATING MORPHOLOGY, MOLECULAR AND ECOLOGICAL DATA TO DELIMIT SPECIES: SUPPORT FOR NEW COMBINATIONS OF *NEOPHYLIDOREA* (DIPTERA: TIPULOIDEA)

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ABSTRACT

Hypothesis testing of species limits is one of the major undertakings in systematic biology. As we have gained analytical tools, data availability and additional resources, multiple means of delimiting species have become possible. However, delimiting species remains a difficult endeavor, because discordance is expected where each criterion has the capacity to identify species limits. This is especially true in recently diverged taxa that may not exhibit features necessary to identify them as distinct lineages. The crane flies (Diptera: Tipuloidea) are a specious group of flies, but little rigorous hypothesis testing has been done to assess the accuracy of species limits. This research explores the use of four methods, each designed to test four newly developed hypotheses based on morphology of a little known genus of flies, *Neophylidorea*. We used a wide spectrum of data including morphology, geometric morphometrics, molecular sequences and ecological niche differentiation to test the four morphospecies hypotheses using a variety of multivariate methods including character and tree-based approaches. Although there were some discrepancies among the datasets and methods with regards to species limits, the consensus suggests that all morphospecies represent independent lineages. Because an overwhelming amount of evidence drawn from a variety
of biological sources was used, we gained a greater understanding of the likely reasons for discrepancies among datasets such as historical hybridization and incomplete lineage sorting.

**KEYWORDS**: crane flies; ecological niche modeling; geometric morphometrics; molecular phylogenetics; species delimitation

One of the major goals of systematic biology is to delimit species. Although operationally delimiting species is considered a “Renaissance issue” in systematics (Sites and Marshall 2003; Sites and Marshall 2004; Wiens 2007), it still takes a back seat to the more pervasive topic in systematic biology of reconstructing phylogenies (Wiens 2007). Although methods for objectively delimiting species have been suggested by a number of authors (Wiens and Servedio 2000; Coyne and Orr 2004), these methods are rarely implemented or explicitly stated (Wiens 1999). Traditional taxonomists often rely on conventional wisdom or expertise, based primarily on morphology to determine the limits of species. But molecular data is increasingly used as a framework for identifying patterns within morphological variation and to identify cryptic species (e.g., Sanders et al. 2006; Condon et al. 2008). These examples provide additional support for the necessity to use multiple lines of objective data to delimit species where one method or criterion can help inform others. Systematics, as an independent science, must take advantage of the plethora of tools and methodology available for delimiting species.

Conflict over species concepts have been debated for decades. The recent shift has been to focus more on the empirical methods used to delimit species rather than the theoretical concept of defining species (deQuieroz 2007). There is ever-increasing impetus for the use of multiple, independent lines of data necessary to delimit species appropriately (Sanders et al. 2006; Roe and Sperling 2007; Leaché et al. 2009, Lumley and Sperling 2010; Ruiz-Sanchez and Sosa 2010). This shift in emphasis somewhat removes the tendency to argue over concepts and to focus more on operational criteria and evidence for species limits. For example, a method involving analysis of phylogenetics would be designed to test evolutionary divergence, whereas criteria utilizing some
ecological differences among species would test niche divergence. Although different disciplines
tend to focus on what methods meet their respective needs, some consensus among methods may
be achieved by understanding the reasons for discordance.

Diverse techniques have recently been introduced (Knowles and Carstens 2007, Raxworthy
et al. 2007) allowing new data types to be incorporated into testing species hypotheses. Various
types of data have been used to delimit species, including unique analyses of morphology (Wiens
and Penkrot 2002; Sanders et al. 2006; Joly and Bruneau 2007; Ross et al. 2010), morphometrics
(Mutanen 2005; Roe and Sperling 2007; Leaché et al. 2009), molecular data (Wiens and Penkrot
2002; Roe and Sperling 2007; Leaché et al. 2009; Leaché and Fujita 2010; Ross et al. 2010; Ruiz-
Sanchez and Sosa 2010) and more recently ecological data (Raxworthy et al. 2007; Rissler and
Apodaca 2007; Roe and Sperling 2007; Leaché et al. 2009; Ross et al. 2010; Ruiz-Sanchez and
Sosa 2010).

Species delimitations are made more difficult when criteria support discordant species limits
(Wake 2006). Different types of data are unlikely to result in equivalent species limits. Ambiguities or
discordance between datasets or analytical procedures can result from incomplete lineage sorting,
historical hybridization, sampling inadequacies, differences in levels of variability, and the overall
ability for a given criterion or method to delimit species in concordance with the timing of divergence
(de Queiroz 2007). Moreover, recently diverged taxa can be among the most difficult taxa to delimit
because they may not have had sufficient time to display divergence and the identification of limits
may especially depend on the methods or criteria used (Shaffer and Thomson 2007). A consensus
approach using many independent lines of evidence used to test species hypotheses is considered
one of the most robust methodology to date (Sites and Marshall 2004; Hey 2006).

Delineating species is important in any aspect of revisionary systematics, but may be
especially important for those groups where original species hypotheses have not been revised.
Crane flies are a specious group of flies (>15,000 species worldwide; Oosterbroek 2010) belonging to
the superfamily Tipuloidea where revisionary work is in its infancy for many groups. The newly
described genus, Neophylidorea is no exception. Neophylidorea was described recently as a
monophyletic genus consisting of 16 species (Chapter 2). A revision of the species within this genus was initiated by studying the majority of *Neophylidorea* type specimens, a wide spectrum of museum specimens and original descriptions. We discovered that delimiting undetermined specimens based on characters provided in the original descriptions was difficult if not impossible due to overlapping intraspecific or clinal variation among identified and unidentified specimens. Much of the morphology used to originally differentiate among species of *Neophylidorea* involved combinations of characters such as minor details of coloration, ambiguous differences such as "long" vs. "short", morphological differences that appear to be related to the method of preservation (e.g., slide mounting causing distortion) or wing patterning. The specific combinations of characters do not always hold true for specimens other than the types. Many species were described in part based on perceived geographic segregation by the original author (Charles Alexander in most cases) but, when a broad spectrum of specimens was considered, the variation among species was continuous. Therefore, our preliminary revision of *Neophylidorea* grouped 16 originally described species into 6 morphospecies based on aspects of gross morphology of the male genitalia and geography. Two additional morphospecies are known from a limited number of preserved samples (<3 specimens in total) and are not considered here. This preliminary revision significantly improved the capacity to delimit morphologically identifiable groups, but still resulted in interspecific and intraspecific morphological variation of characters other than those used to delimit morphospecies. Our objective is to test for significant differences among the four hypothetical morphospecies using a variety of methods and, where the methods permit, to assess any suggestion of cryptic speciation.

Four types of data will be used, each in a variety of ways to test the four morphospecies hypotheses of *Neophylidorea* including detailed morphology, geometric wing morphometrics, molecular sequences, and ecological niche differentiation to delimit species. In this study, we test our morphospecies hypotheses derived from our preliminary revision and presented in greater detail below, using a variety of data and methods. Morphology is used to identify discrete characters and their level of fixation within species. Geometric morphometrics are used to assess differences in wing shape and the ability of this type of data to delimit species where variation may be high but visibly or
qualitatively appears to lack any definite patterns. Genetic data are used to assess the level of exclusivity among morphospecies with respect to mitochondrial and nuclear datasets and to test DNA barcoding methods, or threshold divergence. Lastly, ecological niche differentiation is tested between morphospecies to identify the level of ecological divergence. The consensus of the resulting data will be discussed.

**MATERIALS AND METHODS**

*Morphospecies Hypotheses*

The species hypotheses tested with each of these approaches were based on a combination of macroscopic morphology of the male genitalia and geography. Two morphological features of the adult male consistently distinguish morphospecies from one another, including the shape of the ventral paramere (part of the aedeagal complex) and the median lobe of the 9th tergite. The four morphospecies will be referenced as *adusta*, *caudifera*, *columbiana* and *flavapila* throughout the text. These names are based on priority of the previously described species of which each is comprised (see Appendix 1). Two morphospecies are distinguished based on geography alone (*adusta* is distributed in the east and *columbiana* is distributed in the west (Figure 1) and prior to this research were not known to differ based on morphology.

Two additional species of *Neophylidorea* are not considered here because of low specimen sample sizes. *Neophylidorea vanronae* is known only from two specimens from the same locality, and *N. neadusta* is known only from a single specimen. Both of these species were considered distinct during the preliminary revision based on the ventral paramere shape as well as a combination of other male genitalic characters.

*Research Specimens*

Specimens used in the following analyses were derived from a variety of sources. With one exception, all specimens surveyed were adults. Approximately 500 *Neophylidorea* specimens from museum collections were examined for potential use in delimiting species. Most museum specimens
were dry preserved and pinned or pointed. Male specimens often had genitalia removed and preserved in glycerin in a microvial or slide mounted in Canada balsam. Over 100 additional specimens were collected during trips in 2007 (western North America), 2008 (western North America) and 2009 (eastern North America) (Figure 1; Table 1). Field collected specimens were swept from vegetation and direct killed into 95% ethyl alcohol (EtOH) and stored at -80°C in the laboratory for use in molecular assays. Some specimens were collected into ethyl acetate and dry stored in glassine envelopes and pointed as vouchers. Specimens collected through the course of this research are deposited at the Iowa State Insect Collection, Ames, Iowa, USA and the National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA.

As a means of connecting the following morphological, molecular, and ecological datasets we attempted to collect specimens from the type localities of most described species of *Neophyliidorea*. However, this proved difficult due to the lack of specificity of the type specimen(s) (e.g., *N. tepida* Alexander holotype was collected from “Colorado”) or potentially altered habitat from the original collection locality (e.g., *N. nevadensis* Alexander was collected near Lake Tahoe, Nevada).

As these four morphospecies rely heavily on male genitalia, identifications of females were difficult or impossible. In general, where females were used in the following tests, their morphospecies identity was by association of males collected sympatrically in terms of collection date and location. If male specimens of more than one species were sympatric, female specimens from the same location were excluded from the dataset. Likewise, females collected without associated males were excluded from the following datasets due to the inability to identify with confidence the morphospecies. In the case of molecular sequence data, two females, one from each of the previous categories (sympatric males of two species and lacking any sympatric males) were collected, sequenced and analyzed along with the remaining individuals.

*Morphological Methods*

Morphological tests pertaining to the morphospecies hypotheses were based on two methods. Both methods used 19 categorical morphological characters (Table 2) derived from
previous work identifying morphological variance (Chapter 2) and original species descriptions. We performed a hierarchical cluster analysis, using Ward’s linkage, to create a dendogram with individuals as the terminals. This analysis was performed for male specimens alone. A separate analysis was performed using the entire dataset (males and females) and results are included in Appendix 2. Following the methodology of Wiens and Penkrot (2002), we used a tree-based method to assess species limits using the dendogram and evaluated concordance of the four morphospecies hypotheses with groups of individuals. Although the Wiens and Penkrot (2002) method assessed exclusivity based on populations as terminals, we used individuals as the terminal taxa due to likely polymorphisms within populations. Analyses were completed in JMP version 8 (2009).

We also used a character-based approach to identify the probability that seemingly fixed differences in morphology between species pairs are likely distinct (Wiens and Servedio 2000). This approach determines the statistical significance that at least one character is fixed (intraspecifically invariant) at a frequency of less than 10% based on the specimen sample size (n), the total number of characters (c=19) and the number of seemingly fixed characters (k). The null hypothesis was that polymorphic character states in seemingly fixed characters were present in the species of interest at a rate greater than 10%. We used the supplementary tables provided by Wiens and Servedio (2000), which are based on a binomial distribution, to determine the level of significance for each pair-wise comparison.

**Geometric Morphometrics Methods**

The entire right wing of all available pinned or pointed specimens, with a relatively flat wing and without obvious deformations were positioned with the wing parallel to the surface and photographed. Photos were taken on a white background at 20X resolution with a SPOT RT camera mounted on a Nikon dissecting microscope and taken by the same observer in one sitting. Twenty-five homologous landmarks pertaining to wing venation were digitized (Figure 2) using tpsDig version 2.11 (Rohlf 2008). To eliminate all non-shape variation, the landmark coordinates were subjected to generalized least squares (Procrustes) superimposition (Rohlf and Slice 1990) using tpsSuper
version 1.14 (Rohlf 2004) which superimposes specimens to a common coordinate system after accounting for differences in position, orientation, and size.

Two methods were then used to assess species limits. A Discriminate Function Analysis (DFA) was used to compute morphological variation among morphospecies using all 46 relative warp scores. To quantify the amount of morphological variability of each morphospecies, disparity was measured using the algorithm developed by Foote (1993) and the program DisparityBox6 (Sheets 2003). Multivariate analysis of variance (MANOVA) was conducted on all morphospecies pair-wise comparisons, using all 46 relative warp scores to assess the statistical significance between group means. Statistical analyses (DFA and MANOVA) were conducted using JMP version 8 (2009).

**Molecular Sequencing**

A total of 57 populations of *Neophrildorea* were sampled in 2007 (western USA), 2008 (western North America) and 2009 (eastern North America and one California population). Whole genomic DNA was extracted from individual specimens using the DNeasy Tissue Kit (Qiagen) under standard protocols, except that samples were eluted using 100 µl of buffer twice. Multiple techniques involving different combinations of body parts were tested to determine the combination that preserves the specimen and the morphology. The best method involved using the entire, intact insect in the extraction. Specimens from which DNA was extracted were maintained as voucher specimens. The final concentration of DNA was diluted to 1:10 using DNA free water for use in PCR.

We sequenced the complete mitochondrial (mtDNA) cytochrome oxidase subunit I and II genes (COI and COII), often referred to throughout the text as the mtDNA dataset. A subset of individuals was selected to represent the full spectrum of mtDNA haplotypes and a portion of the nuclear gene rudimentary, or carbamoyl phosphate synthase (CAD) was sequenced from these individuals. The fragment sequenced included parts of segments two and three as described by Moulton and Wiegmann (2004). The mtDNA genes are commonly used in investigations of species limits (e.g., Hebert et al. 2003; Lewis et al. 2005) and in insect phylogenetics specifically (Caterino et al. 2000). The nuclear gene, CAD, employed here has been suggested for use in delimited species
(Moulton and Wiegmann 2004), but rarely implemented at the species-level (other than Maddison and Arnold 2009).

Three “Limnophilinae” outgroup taxa were sequenced for COI and COII: *Epiphragma solatrix* (Osten Sacken), *Prionolabis politissima* (Alexander), and *Euphyllidorea platyphallus* (Alexander). A slightly different set of outgroup taxa were chosen for CAD due to the difficulty in amplification of this gene: *Epiphragma solatrix*, and *Euphyllidorea similis* (Alexander). These outgroups were chosen to represent some of the most likely closely related species (e.g., *Eu. similis* and *Eu. platyphallus*) as well as some more distantly related groups (*Ep. solatrix* and *P. politissima*) based on a recent phylogenetic analysis of crane flies (Petersen et al. 2010).

PCR and sequencing primers are shown in Table 3. PCR was run in 10 µl amplification reactions. Each reaction contained 1 µl of 1:10 diluted genomic DNA, 10 µM Tris-HCl (pH 8.0), 50 µM KCl, variable MgCl$_2$ (COI, CAD: 2mM; COII: 3 mM), 0.25 mM of each nucleotide, 0.25 mM of forward and reverse primers, and 0.025 U of Taq Jumpstart polymerase (Sigma). Negative and positive controls were included in all sets of PCR. Thermal cycling profiles varied but in general consisted of an initial denaturing at 95˚C for 5 min; 32-35 cycles of denaturing at 95˚C for 60 s, annealing at 58˚C (COI, COII) or 48˚C (CAD) for 60 s, and extension at 72˚C for 2 min; and a final extension at 72˚C for 5 min. Thermal cycling and subsequent cycle sequencing was conducted in PTC-220 Dyad Thermal Cyclers (MJ Research).

We electrophoresed 2 µl of the PCR products in 2% agarose TAE gels with ethidium bromide staining to confirm amplification and fragment size. To digest primers and unincorporated nucleotides, we added 0.5 U Exonuclease (USB) and 0.5 U Shrimp Alkaline Phosphatase (USB) to the remaining 8 µl of each PCR product. These samples were incubated for 30 min at 37˚C, then 10 min at 90˚C. COI was sequenced using four primers. Cycle sequencing was conducted using amplification primer for all loci and internal primers for COI (2183 and 2191) to ensure complete coverage of the gene. We used BigDye 3.1 (Applied Biosystems) chemistry with the recommended cycling conditions, and sequences were read using Applied Biosystems model 3730 automated DNA sequencers. Nearly all fragments were confirmed by sequencing both strands. Sequences were
checked, concatenated, and alignments were made using the program Sequencher 4.5 (Genecodes). Sequences were subjected to BLAST analysis and checked for any premature stop codons to assess pseudogene presence.

*Molecular Delineation Methods*

A variety of methods were used to delimit species based on molecular sequences. First, a tree-based method was used independently for the mtDNA and nDNA datasets. Next, nested clade phylogeographic analysis, a method typically used for phylogeographic investigations, was implemented here as a means of providing additional insight into the evolutionary processes of divergence that may have lead to lineage formation (Wiens and Penkrot 2002). Lastly, standard DNA taxonomy or DNA barcoding techniques were used to test species limits and are compared to the morphospecies hypotheses.

Mitochondrial sequence data were analyzed as a concatenated dataset. Akaike information criterion (AIC) was used to select an evolutionary model that best fits the observed data using the program jModeltest (Posada 2008). The model GTR+G+I was incorporated into a maximum likelihood analysis using RaxML version 7.0.3 (Stamatakis 2006) as a tree-based method to infer species boundaries. Statistical branch support was evaluated with 100 bootstrap replicates. The tree-based method developed by Wiens and Penkrot (2002) was used to assess species limits using the mitochondrial and nuclear datasets independently.

For support of the previous test, and as suggested by the above authors (Wiens and Penkrot 2002), nested clade phylogeographic analysis (NCPA; Templeton et al. 1995, Templeton 1998) was performed using ANeCA version 1.2 (Panchal 2007). This program was used in coordination with TCS version 2.1 (Clement et al. 2000), which built the haplotype network, and GeoDis version 2.5 (Posada et al. 2000), which implemented NCPA and the inference chain (Templeton et al. 1995). Nested clade phylogeographic analysis was performed only on the more robust mitochondrial dataset using latitude-longitude coordinates to infer geographic distances. Haplotypes were analyzed using the default settings in TCS, identifying networks through parsimony at a 95% connection limit.
was also run and NCPA was performed using a 97% connection limit. This higher connection limit ensured that various groups of interested were considered within the same clade. The radius used for all populations was set at 3 km, which was chosen to match approximate sampling intensity. The inference chain (IC) was inferred from the ANeCA results to interpret the statistical results of the NCPA in the framework of delimiting species. This method is known to produce false-positive results under various simulation scenarios (Knowles and Maddison 2002; Panchal and Beaumont 2007; Knowles 2008), although some authors have refuted the complaints against NCPA (Templeton 2008, 2009). Our results are interpreted with caution and this method is one of many lines of evidence.

DNA taxonomy, or barcoding methods, as criteria for delimiting species vary greatly among researchers (Meier et al. 2006). For simplicity, we chose a single tree-based method using parsimony assessed at a 95% connection limit using COI (Posada and Crandall 2001; Hebert et al. 2003). That is, species are determined to be distinct at a genetic divergence of 5%. This analysis was performed using TCS version 2.1 (Clement et al. 2000). The results are compared with respect to morphospecies identity. Because of the current interest in barcoding methods, alternative criteria were also assessed following Meier et al. (2006) and using the associated program, TaxonDNA. These criteria included assessing the presence of a barcoding gap, or a gap in the frequency distributions between intra- and interspecific uncorrected pair-wise distances; detecting if the “best match” for each individual based on the smallest genetic distance is a conspecific; detecting the “best close match” based on the calculated threshold of intraspecific divergence; and identifying whether all “best matches” of a particular individual are conspecific, or “all species barcodes” (Meier et al. 2006).

Ecological Niche Modeling Methods

Two analyses were conducted to identify differences in the ecology between morphospecies. Both analyses included identifying known locations of populations for each morphospecies and assessing differences in the ecological niche based on broad, abiotic environmental data. Environmental variables included 19 bioclimatic variables from the Worldclim dataset (Hijmans et al. 2005) and compound topographic index (CTI) which indicates wetness (courtesy of the U.S.
Geological Survey), at a spatial resolution of 2.5 arc-minutes. These variables were chosen to represent a broad spectrum of environmental factors (precipitation, temperature, wetness) that are likely to play a role in determining the potential distribution of *Neophylidorea* species due to their affinity for high altitude (cold) and wet (precipitation and wetness) environments (Chapter 2). First, a principal component analysis (PCA) was conducted to visualize the differences in their environments from known localities. The second method involved modeling the ecological niche, by first statistically testing the degree of niche overlap between all pair-wise comparisons and then considering the background from which the morphospecies are derived.

For the PCA, 1000 random background points were derived based on the minimum convex hull surrounding all known occurrences of each morphospecies. Together with the actual occurrences, data from the 19 Worldclim layers and CTI was extracted from these 1000 points using ArcGIS 9.2 (ESRI 2009). PCA was conducted using environmental data extracted from all known localities and 1000 random background points for each morphospecies and visualized using the program JMP version 8 (2009).

Ecological niche modeling (ENM) was performed using Maximum entropy modeling through the program Maxent version 3.3.1 (Phillips et al. 2004; Phillips et al. 2006; Phillips and Dudík 2008). Maxent was chosen here because in comparative analyses it has consistently outperformed other modeling algorithms (Elith et al. 2006; Hernandez et al. 2006). This method uses iterative correlations with presence-only occurrence data to build the model. Due to low sample sizes (N: *adusta* = 72, *columbiana* = 39, *flavapila* = 51, *caudifera* = 16) final models were averaged over 20 runs.

ENMtools was used to test differences in niche identity and background overlap between all pair-wise comparisons of morphospecies (Warren et al. 2008; Warren et al. 2010). We tested niche overlap using the niche identity (*I*) statistic developed by Warren et al. (2008), which ranges from 0 (no overlap) to 1 (identical). Specifically we tested all pair-wise comparisons using randomization procedures of 100 pseudoreplicates for both the niche identity test and background tests described by Warren et al. (2008, 2010).
For each pair-wise comparison, the actual niche identity ($I$) was calculated by first developing an ENM for each species using Maxent, then calculating the statistic in ENMtools. Subsequently, pseudoreplicates were created by randomly assigning morphospecies identity to each locality in the comparison, ending with sample sizes equal to the actual comparison of interest. For each replicate, just as in the actual comparison, ENM’s were created and the niche identity was calculated. The distribution of pseudoreplicates was compared against the actual niche identity value to test whether the actual niche is significantly different from randomly generated measures. The hypothesis of niche identity is rejected if the actual observed measure of $I$ is significantly lower than the null distribution.

Background overlap tests were conducted by calculating the ENM’s and associated $I$ statistic for each pair-wise comparison but this time using actual occurrences for one species and random occurrences of the original sample size, drawn from the original background for the other species. The background overlap tests were conducted in both directions (actual occurrences of morphospecies A compared to random background occurrences of B and vice versa). Again we generated a null distribution in both directions, both with 100 pseudoreplicates. These null distributions were compared with the actual $I$ values for each pair-wise comparison. For the background overlap test, the hypothesis is two-tailed. In other words, a pair of morphospecies can be more or less similar to each other than expected.

The extent of the background from which the tests of niche identity are drawn is important to consider (Warren et al. 2010). The minimum convex hull surrounding known localities was used as a mask for niche identity and background overlap randomization tests specific to the comparison being made. Six total comparisons were made (all pairs of morphospecies), therefore six masks consisting of the minimum convex hull for each pair-wise comparison plus separate masks for each morphospecies alone were developed using ArcGIS 9.2 (ESRI 2009).

RESULTS

Morphological Results
A total of 257 individuals were investigated for the morphological hierarchical clustering analysis. Visually, the clusters correspond to the morphospecies hypotheses (Figure 3), with a few exceptions. The results were derived from all 19 variables, but only a few characters were fixed among morphospecies. The morphospecies *adusta* and *columbiana* did not form discrete clusters based on this dataset and analysis. Five individuals of *columbiana* clustered with individuals of *adusta*, based on a shared presence of apical wing clouding (character 15). These *columbiana* individuals were from a population in northwest Wyoming, at the eastern edge of its distribution and were drawn from a population where apical wing clouding was polymorphic. Some individuals from this same population did not have apical wing clouding, and clustered with other individuals identified as *columbiana*. Because geography was used as a means of identifying morphospecies, all specimens from Wyoming were identified as *columbiana*, regardless of wing patterning. Results were based exclusively on male specimens due to a variety of characters dealing with male genitalic features (characters 1-3, 18-19). The results of the same analysis including male and female specimens (N=416) and only characters 4-17, indicated a complete lack of clustering corresponding to morphospecies (see Appendix 2).

Using the tree-based method developed by Wiens and Penkrot (2002), most morphospecies were considered exclusive with regards to the hierarchical clustering dendogram. The only morphospecies that were not exclusive were *adusta* and *columbiana*. Five individuals of *columbiana* that clustered with *adusta*. As suggested above, there was no geographical structure to those non-exclusive individuals. The remaining morphospecies, *flavapila* and *caudifera* represented exclusive species.

The character-based test developed by Wiens and Servedio (2000) showed that nearly all morphospecies comparisons possessed at least one fixed character as determined by their sample sizes, the number of apparently fixed characters and number of characters surveyed (c=19) at a probability or frequency cut-off of 0.10 (Table 4). The only exception involved comparisons made between all morphospecies and *caudifera*. Therefore, we rejected the null hypothesis of the
presence of rare character states in seemingly fixed characters in all pair-wise morphospecies comparisons with the exception of caudifera.

**Geometric Morphometrics Results**

Wings from a total of 406 individuals were digitized for 25 landmarks (Figure 2). Of those, 37 individuals were misidentified using DFA. Of the 151 individuals identified as adusta, 88.7% were correctly identified as such. The majority of misidentified individuals were classified as caudifera. Surprisingly few were misidentified as columbiana. Of the 24 caudifera individuals, 87.5% were correctly identified and all three misidentified individuals were classified as adusta. Many individuals of columbiana were classified correctly (90.3%) with majority of those misidentified being classified as adusta. Few individuals of flavapila were misidentified (4.7%) and this morphospecies had the largest disparity of all four morphospecies.

Based on the results of MANOVA, all pair-wise comparisons were significantly different from each other (p<0.0001), indicating that the mean relative warp scores for the first two axes differed for all morphospecies (see Appendix 3). The average coordinates of each morphospecies (Table 5), were distributed one in each of the four quadrants of the first two relative warp axes. However, visualization of the deformation grids for each average morphospecies showed few differences between species with regards to the first two relative warp axes. The only differences were with respect to the Rs vein, or the vein connecting landmarks 1 and 2 (Figure 2). In caudifera specimens, Rs was considerably shorter (landmark 1 is closer to landmark 2) and flavapila specimens had a considerably longer Rs vein (landmark 1 is farther away from landmark 2) with respect to the average wing from all specimens. Average adusta and columbiana wings vary little from the entire group average.

**Molecular Sequence Results**

A total of 2196 base pairs of COI and COII were sequenced from 81 individuals including three outgroup taxa (Table 1). A total of 458 base pairs were parsimony informative. Following
removal of identical haplotypes, the final dataset included 63 ingroup taxa. The majority of individuals removed were due to duplicate haplotypes from individuals within a population (B0207, T2307, T1808, T5407, T5707, and T5907). However, some taxa with identical genotypes consisted of individuals from different populations (B4407 and B4207; T1608, T1808; B1408 and B5707; A12309, A12409, and A9909; A10109, A3909, A7309 and A9809).

A total of 1056 base pairs of CAD were sequenced from 22 individuals including 58 parsimony informative sites (Table 1). This gene was more difficult to amplify than COI and COII and as a result, fewer individuals were sequenced. All individuals sequenced represented unique haplotypes.

Phylogenetic gene trees produced from a maximum likelihood analysis for both the mtDNA (Figure 4) and nuclear (Figure 5) datasets were used to infer species boundaries based on the tree-based method outlined by Wiens and Penkrot (2002). For the mtDNA dataset, caudifera was the only morphospecies that was considered a single, exclusive species. Morphospecies flavapila was non-exclusive due to the T2307 population collected from eastern California, resulting in two cryptic species. Both adusta and columbiana were non-exclusive as focal species and conspecific with each other. The non-exclusivity between adusta and columbiana was due to a clade consisting of B1708 (columbiana) collected from Idaho and A9509 (adusta) collected from Maine.

The gene tree produced by CAD indicated slightly different results (Figure 5) than that produced from mtDNA. The morphospecies caudifera was represented by a single individual in this dataset and therefore its identity could not be tested using this tree. Contrary to the mtDNA results, this tree indicated strong support for an exclusive flavapila clade (BS = 0.96), consisting of the previously non-exclusive T2307 population from California and two additional populations (T5407 and T1208) from Oregon and Idaho respectively. Similar to the mitochondrial results, adusta and columbiana were considered non-exclusive and conspecific to each other. The relatively strongly supported clade (BS = 0.78) that lead to non-exclusivity consisted of four populations, widely separated geographically: columbiana populations B1708 (Idaho, same as mtDNA) and B0207 (Wyoming), and adusta populations A9509 (Maine, same as mtDNA) and A9809 (Nova Scotia).
Nested clade phylogeographic analysis on mtDNA produced eight networks at the 5% threshold and seven networks at a 3% threshold. The merged networks as a result of lowering the connection limit included all *adusta* individuals and a single population of *columbiana* (B1708; clade 6-5 in Figure 6). Numbering of clades accounted for empty clades and not all clades or haplotypes are identified. Network 5-8 consisted of entirely *columbiana* individuals, separated into multiple clades within (Figure 7). Morphospecies *flavapila* consisted of four total networks: 5-2, 5-4, 5-6 and 5-7 (Figure 8), three of which were represented by a single population. Lastly, the two individuals of *caudifera* formed a separate network (Figure 8).

The analysis showed a lack of statistical significance for most clades with respect to geographical association (Table 6). The only clade corresponding to morphospecies for which there was any support was the *flavapila* (clade 5-2), minus the single population from California (T2307). The chain of inference suggests restricted gene flow with isolation by distance. Two additional clades indicated allopatric fragmentation (3-11 and 3-12), but did not correspond to morphospecies.

Lastly, DNA barcoding results were not illustrated here due to the similarity in the haplotype network formed by using COI alone compared with the entire mtDNA dataset used for the NCPA. The analysis with COI alone resulted in seven discrete networks. There was only a single difference in terms of the general networks formed from COI alone, as compared with the entire mtDNA dataset at the 5% divergence threshold. The *flavapila* individual, T1208a collected from northwest Wyoming did not form its own cluster (as in Figure 8), but clustered with the larger *flavapila* network (network 5-2 in Figure 8). The population from eastern California, T2307 still formed its own network as well as one of the many populations from Idaho, T1708 as a unique network. Populations of *adusta* (A9509) and *columbiana* (B1708) continued to form a distinct cluster (network 5-3 in Figure 6). The remaining individuals of *adusta, columbiana, flavapila* and *caudifera* formed their own independent networks. From this analysis *caudifera* is the only morphospecies that did not indicate some level of cryptic speciation. Results of various other barcoding criteria are included in Appendix 4. In summary, DNA barcoding results suggest seven species including cryptic speciation in *flavapila* (three species in
total) and a cryptic species comprised of individuals identified as morphospecies *adusta* and *columbiana*.

**Ecological Niche Identity Results**

A total of 173 known occurrence localities across all four morphospecies were used in niche based criteria of species limits. Principal components 1-3 explained a total of 80% of the variation. The components were difficult to generalize in terms of variables loadings on each component (Table 7). In other words, no environmental variable had high loadings on any of the first few principal components. The PCA showed some separation of the morphospecies based on 19 bioclimatic variables and compound topographic index (Figure 9). A visual inspection of PC1 against PC2 indicated little clustering of background points or known localities of morphospecies. Morphospecies *columbiana* and *flavapila* formed somewhat disjunct clusters, but there was no geographical correspondence to these within morphospecies groupings. PC1 against PC3, and PC2 against PC3 showed separation of both the background points and the known localities based on morphospecies identity. Both comparisons indicated similar niche and background environment between *adusta* and *caudifera*, and *flavapila* and *columbiana*.

By inspecting the loadings of environmental variables (Table 7) in combination with the score plot (Figure 9) we identified parameters that are associated with various groupings of points. Those environmental variables associated with the *adusta-caudifera* cluster included almost all precipitation parameters (warmest quarter, wettest quarter, wettest month, annual precipitation, driest quarter, and driest month) and temperature seasonality. In contrast, few environmental variables were associated with the *flavapila-columbiana* cluster: precipitation of the coldest quarter, isothermality, and mean temperature of the driest quarter. Known localities of each morphospecies were nested within their respective background points. Background points of each morphospecies overlapped significantly with other morphospecies, while known localities did not. For example, *adusta* background points were distributed throughout the range of *columbiana* and *flavapila*, despite little overlap of the known occurrences of *adusta* in comparison to *columbiana* and *flavapila*. 
Randomization tests of niche identity indicated all pair-wise morphospecies comparisons were ecologically independent (P<0.01) with the exception of *flavapila-columbiana* where we failed to reject the null hypothesis that niches are identical (Table 8). These results are consistent with the fact that *flavapila* and *columbiana* are often found sympatrically at the site level, while all other morphospecies are either allopatric or regionally sympatric, but not found at the same local site. The niche identity test may not be the most appropriate test for allopatric species because it does not take into consideration the differences in the environment in which morphospecies inhabit. Therefore, background overlap randomization tests were conducted on all pair-wise comparisons (see Appendix 5 for frequency histograms of niche identity and background tests).

The results of background overlap tests are best understood by taking the level of range overlap into consideration (i.e., allopatric versus sympatric distributions). Comparisons made between sympatric taxa (*adusta-caudifera* and *flavapila-columbiana*) suggested that morphospecies were more similar than expected by chance when the background in which they occur was considered, in at least one direction (i.e., *columbiana* was more similar to a model based on random background points from *flavapila* background, but not in the other direction; Table 8). When *adusta* was compared with either allopatric morphospecies *columbiana* or *flavapila* the results indicated distributions were less similar than the actual niche identity. Background tests with *caudifera* compared to both *flavapila* and *columbiana* were more complex. In one direction, the morphospecies were less similar than expected based on the background of one species, but more similar based on the background of the opposite comparison. In both instances, the analysis based on the *caudifera* background produced the more similar than expected result indicating greater environmental heterogeneity of this morphospecies. This is consistent with the fact that the range size of *caudifera*, based on the minimum convex hull surrounding known localities is large compared with other species.

**DISCUSSION**
We produced a thorough, integrative taxonomic approach to delimiting species of a little known group of flies. All four datasets and associated analyses presented here are, in general, in concordance with regards to validating the original morphospecies hypotheses. Where encountered, discrepancies were generally not consistent across methods and are likely due to different criteria assessing or detecting different stages (Nosil et al. 2009) or levels of speciation. This research demonstrates the need for multiple, objective lines of evidence when delimiting species because of the various discrepancies. Because multiple methods were utilized for each dataset, we took a unique, comparative approach to determine which methods may be identifying errors in species limits. Morphology provided some of the most straightforward and applicable results both in terms of discrete morphological characters and geometric morphometrics. Genetic data produced inconsistent and potentially misleading results if not interpreted with caution. Lastly, the results of ecological niche identity and background tests were particularly interesting where significant niche differentiation was found in likely, recently diverged taxa.

The results from multiple analyses within a particular dataset are often strikingly dissimilar. However, careful investigation of the potential for false-negative and false-positive results indicates the pitfalls of various criteria. Based on the lineage concept, no single method can be validated for each species to delimit taxa (de Queiroz 2007). Rather, the goal is to seek corroboration from multiple methods. Because there is no reliance on a single method, and all methods are treated equally, we chose to analyze each dataset in multiple ways to again build as much evidence as possible. Similarly, requiring strict monophyly or complete reproductive isolation would undermine the divergent properties that have created the diversity we see today. The consensus from all methods suggests that despite potential ongoing gene flow between some taxa, all morphospecies should be revised and redescribed as valid species.

*Morphology*

Traditionally, morphology has been used exclusively as a means of delimiting species, although usually not very objectively or with much statistical rigor. Various multivariate methods
identifying clusters or groups using continuous and discrete morphological data have been implemented with a high degree of success (Henderson 2002; 2004; Sanders et al. 2006). The clustering analysis used here is concordant with the morphospecies hypotheses and generally with the other datasets. Although the cut-off for defining species could be drawn at different levels in the hierarchical clustering dendogram (Figure 3), we were most interested in whether our morphospecies were validated through this analysis. No large groups of individuals were formed that included at least one fixed character below the morphospecies-level. These results indicate that no distinct lineages exist below the morphospecies-level (i.e., no cryptic speciation). Hierarchical clustering was important in demonstrating, at least visually, that much of the variation used to originally describe species lacks pattern or structure. Overall, this multivariate method could be helpful in the early stages of forming species hypotheses and identifying seemingly fixed characters. Through this method we identified a seemingly fixed character that had previously gone undetected (e.g., wing clouding at base of Rs between adusta and columbiana).

The character-based analysis indicated that although most morphospecies comparisons are discriminated using few characters, based on the sample sizes used here, at least one character is seemingly fixed between all morphospecies comparisons. The statistical significance of the character-based analysis overshadows the non-exclusivity in the tree-based methods. One of the primary reasons for lumping previously described species into the morphospecies hypotheses tested here, was because of the high degree of variability within and between species. Only one species was not discriminated using the character-based approach. This is due to the low sample size of caudifera (n=17) and few seemingly fixed characters (k=2 to 3; depending on the pair-wise comparison).

Although stressing the need for multiple means of delimiting species is important, morphology is still the most commonly used method to identify species. Particularly in insects, aspects of genitalia, which represent the majority of the fixed characters, are thought to define reproductive isolation (Eberhard 1985). Therefore, this result is nontrivial and of primary importance with regards to the ultimate limits of species.
Geometric Morphometrics

Traditional morphometrics have long been used to delimit various aspects of insect biology (Daly 1985) including delimitation of species, but only rarely are geometric morphometrics used today to delimit species (Dujardin et al. 2003; Mutanen 2005). Geometric morphometrics captures all aspects of shape variation (for the landmarks under consideration) and is therefore often more informative than traditional morphometric methods (Adams et al. 2004). In many insects, wing shape has the potential to provide a relatively simple means of delimiting species (Baylac 2003; Dujardin et al. 2003). Wing morphology has been used extensively in crane flies to delimit taxa, although it has not previously been quantified in this manner. The Radial sector (Rs) vein was used by Alexander (1972) as a defining character in his description of the genus *Euphylioidorea* (the genus in which species of *Neophylioidorea* previously resided), describing Rs as “short”. A large amount of data on wing shape is relatively easy to collect and can utilize a potentially vast array of museum specimens. In our case, variation among specimens in wing shape was apparent, but was difficult to quantify or describe based on visual inspection. Geometric morphometrics quantified the variation in wing shape and through a classification algorithm, determined the identity of specimens with a high degree of accuracy. Using these data, unknown specimens (especially females or individuals lacking genitalia) can be identified with an accuracy of >90% in most cases. This result was particularly surprising given that prior to this analysis visual inspection of wing shape did not show consistent variation among morphospecies. The results suggest that there is indeed pattern to the wing shape differences, especially with regard to the Rs vein.

Some methods presented here are somewhat circular, in that the output can be dependant on the information provided with the input. For example, discriminate function analysis of the relative warp scores determines group identity based on the original identities proposed. Therefore, the classification rate is biased because the predefined group means are used to develop the algorithm which in turn determines the group identity of all individuals. A high degree of care was taken to ensure that the originally identified individuals were accurately delimited as the appropriate
morphospecies. Where sympatry between species is likely or known to occur, female specimens were matched with supposed conspecific males, or excluded from the dataset.

**Molecular Sequences**

Genetic data has been shown to provide a signal of species-level divergence, and can be particularly useful in delimiting recently diverged taxa (Rosenberg and Nordborg 2002; Degan and Salter 2005; Shaffer and Thomson 2007). These results suggest that molecular data should not be used exclusively, at least using the methods we employed. The results of four methods utilizing different combinations of datasets (mtDNA and nuclear DNA) yielded the most varied and unsupportive tests of the morphospecies hypotheses. Although a lack of support alone is not sufficient to discredit a particular criterion, the results of various genetic criteria presented here suggest cryptic speciation where other evidence is lacking (e.g., geographical structure).

The mtDNA dataset failed to recover a monophyletic or exclusive flavapila clade whereas monophyly was achieved with the nuclear data. This is just one example in a long line of evidence (Will and Rubinoff 2004; Cognoto 2006; Roe and Sperling 2007) that suggests mtDNA may not necessarily be a widespread predictor of species limits. Although monophyly may not be necessary to define species limits (Knowles and Carstens 2007; Weisrock et al. 2010), no other datasets identify cryptic speciation within flavapila. Of the three methods employed here utilizing DNA sequence data, the tree-based method was the most robust, especially when both gene trees were considered simultaneously.

Comparing the gene trees produced from the mtDNA dataset with the CAD dataset suggests that both exhibit relatively high levels of variability between species to capture divergence at this level. Our results confirm that multiple, unlinked genes including nuclear and mitochondrial regions are helpful in sorting out various discordances caused by incomplete lineage sorting or hybridization. Rudimentary (CAD) has been used frequently for higher level phylogenetics (Danforth 2006; Moulton and Wiegmann 2007; Petersen et al. 2010), but this research and others (Moulton and Wiegmann 2004; Maddison and Arnold 2009) identifies its usefulness in delimiting species.
Molecular methods often identify more species than other criteria such as morphology or ecology (Hebert et al. 2004; Weisrock et al. 2010). Threshold based delimitation of species employed here as the barcoding technique using COI clearly identifies the greatest number of total species, with only one morphospecies (*caudifera*) identified as a discrete lineage. However, there is little to no geographic basis for many of what would be considered cryptic species. For example, the *flavapila* population from Idaho, T1708 is clustered in a high concentration sampling area (near T1408, T1608, T1708 and T1908; Figure 1). Many of the alternative barcoding criteria suggested a failure of the methods (Appendix 4), similar to other studies (Meier et al. 2006; Virgilio et al. 2010). Based on the lack of a “barcoding gap” in this dataset, thresholds of intraspecific distances cannot be used to delimit species, again similar to other findings (Wiemers and Fiedler 2007; Meier et al. 2008). Despite the desire for the ultimate, unbiased, and clear-cut estimate of species limits through DNA taxonomy (Hebert et al. 2003; Tautz et al. 2003; Hebert and Gregory 2005), these results and others (Will and Rubinoff 2004; Will et al. 2005; Meier et al. 2006; Wiemers and Fiedler 2007; Spooner 2009) suggest that, although it may be easily accessible, DNA taxonomy overestimates the number of species.

Nested clade phylogenetic analysis has often been criticized for producing false-positives (Panchal and Beaumont 2007). This analysis likely produced false-positives of allopatric speciation within the major *flavapila* network, but could easily be discounted because no other methods support division of this group. The same data were analyzed using a tree-based approach and did not identify the same multiple independent evolutionary lineages within *flavapila* as the NCPA. The more troubling result of the NCPA was the lack of support or inference of allopatric speciation at other levels identified by many other datasets suggests that this method may not be the best approach to defining the limits of species. These results should be interpreted with caution given the lack of data for certain morphospecies such as *caudifera* and inference based on a single type of genetic data (i.e., mtDNA).

*Niche Differentiation*
Dispersal limitation has been described as a potentially important aspect shaping the process of speciation (Mayr 1942, Coyne and Orr 2004). Ecological divergence can cause or contribute to reproductive isolation and provide evidence for delimiting species (Van Valen 1976, Andersson 1990). These results are among the most supportive of the morphospecies hypotheses, suggesting that even closely related species (*adusta* and *columbiana*) inhabit non-overlapping ecological niche space, even when the background from which they are drawn is taken into consideration. Some results indicate morphospecies niches are more similar than expected but involve comparisons made between sympatric taxa. These results do not support lineage differentiation, but they can be discounted as false-negatives because clearly sympatric taxa inhabit similar environments at the spatial scale we tested. The high degree of niche divergence among closely related species is somewhat surprising. Other research indicates that recently diverged taxa should occupy similar niches (Kambhampati and Peterson 2007).

The cluster of *flavapila* specimens from PCA consists of populations collected from Alaska and British Columbia, the far northwest range of the species. This suggestion of cryptic speciation does not correspond to the same geographic localities of the cryptic speciation suggested by NCPA or that of the Sierra Nevada population suggested by the tree-based molecular methods. There is clearly a lot of heterogeneity and diversity in geography, wing shape, genetics within *flavapila*. However, that diversity does not show consistent patterns across methods.

The PCA of known localities and background points was used as an exploratory method to assess potential underlying differences in the ecology of these morphospecies. The lack of high loadings on any particular principal component is likely due to the fact that many of these variables are highly correlated. Although visually interesting, this approach did not produce the biological significance one might wish to gain from its use. Background points of conspecific morphospecies were generally clustered together (i.e., western North America is environmentally distinct from eastern North America). Some environmental variables were correlated with known morphospecies localities, but because the background points also clustered, we cannot separate inferences based on known localities from those just referring to the broader environment from which they are drawn.
Therefore, this approach should be interpreted with caution, but provides an important tool for visually interpreting the results of other niche-based significance tests.

*Indiscrete adusta – columbiana group*

If exclusivity or monophyly is used as the sole criteria for delimiting species, all molecular datasets used indicate a single species group including *adusta* and *columbiana*. Some suggest that distinct species can continue to engage in limited gene flow (Wiens and Penkrot 2002) or that incomplete lineage sorting or other factors leading to lack of reciprocal monophyly are not necessarily reason to reconsider species limits (Knowles and Carstens 2007). Between morphospecies *adusta* and *columbiana*, speciation may not have proceeded as far as the other species considered. Recognizing species in the early stages of divergence is particularly difficult, but perhaps the most rewarding in terms of the mechanisms that potentially lead to their formation. The challenge stems from the fact that patterns are difficult to discern over such short time periods.

Other lines of evidence suggest that *adusta* and *columbiana* represent distinct lineages with some degree of either continued exchange or recent histories of genetic exchange. Morphological data revealed that despite lack of exclusivity using a tree-based method, the character-based test indicated significance of a single fixed feature separating the two morphospecies. All that is needed for most taxonomic investigations a single fixed character (Wiens and Servedio 2000). Few *adusta* wings were classified as *columbiana* (2.6%), but the percentage was higher for the reverse comparison (6.5%). Again, these two morphospecies are not in all measures distinct, but statistically independent enough to be considered separately evolving lineages, or species. Likewise, the ecological data tested for differences between *adusta* and *columbiana* and results showed independence of ecological niches even when background data was taken into consideration.

*Implications for Crane Fly Systematics*

There is a need for statistical rigor in crane fly systematics. With these objective methods, workers can begin revising many groups where species may simply represent clinal variation as in
the present case. Although taxonomically diverse (de Jong et al. 2008), this group suffers from a paucity of biological information. Likewise, few comprehensive revisions have been completed. Molecular sequence data have only been used in a few instances to infer phylogeny (Nitta and O’Grady 2008, Petersen et al. 2010) and both were at the genus level or higher. This is the first research to assess species-level molecular diversity within crane flies.

Perhaps because of the few revisions, the observed synonymy rate (species-level taxonomic hypothesis failure) of 9.7% (number of synonymized species/total number of described species; Oosterbroek 2010) is low compared with other insect groups (Gaston and Mound 1993). Superficially having a low synonymy rate might seem good, but the taxonomy of most species and genera has not been revised. The true level of diversity is of importance for an inherent understanding of the total biodiversity as well as making steps towards a better understanding of their ecological roles.

Although some have suggested that, with an increase in use of molecular resources to delimit species of crane flies, the known species diversity will rise (de Jong et al. 2008), these results indicate that fewer species many, in fact, be justifiable.

A formal revision of Neophylidorea will follow this publication, including the four morphospecies evaluated here as well as two additional species (N. neadusta and N. vanronea) for which there are too few specimens to be included in the present analyses. These species clearly represent distinct, separately evolving evolutionary lineages and therefore are considered species as a result of the multiple criteria and methods used here.

Limitations

One of the downfalls of our genetic data was that only two genes were considered. The use of one or only a few genes may lead to inaccurate inferences regarding species boundaries as there is often a discordance between gene trees and species trees (Pamilo and Nei 1988; Degnan and Rosenberg 2006).

Similarly, mitochondrial DNA has been controversial with advantages and disadvantages proposed for use in delimiting species. These data can be particularly problematic due to possible
incomplete lineage sorting leading to incorrect species delimitations (Funk and Omland 2003). Other authors suggest that mtDNA is particularly useful in assessing species-level relationships because of its rapid evolution, limited recombination and lack of degradation (Avise et al. 1987), and may coalesce quicker because of the smaller effective population size (Moore 1995).

There are geographic limitations with regards to the DNA dataset. Before this research, little was known about habitat requirements of this genus and few specific collection records existed. Considerable effort was taken to collect fresh specimens from across North America. We were unable to cover the entire geographic extent during the course of this research such as areas of Alaska and Colorado. Fresh specimens used for molecular analyses were collected with some degree of spatial autocorrelation which may have lead to some erroneous predictions such as the cryptic speciation result from the NCA. However, these limitations apply only to the molecular dataset and, therefore, reinforce the need for multiple lines of evidence in delimiting species.

Perhaps additional sampling will support some of the evidence for cryptic speciation within *flavapila*. Other researchers have shown lineage divergence at much finer geographic extents (Kuchta et al. 2009; Weisrock et al. 2010). This may be especially true in areas with large potential geographic barriers to dispersal such as the Central Valley of California. Little is known about the habitat requirements of *Neophylidorea* morphospecies, although they tend to inhabit a wide variety of environments from fens to rivers to lakes. Future research may suggest morphologically cryptic species that show concordance with habitat requirements.

**Potential Mechanisms of Evolution**

Allopatric speciation, or isolated geographic distinction likely played a role in forming species within *Neophylidorea*. This is perhaps the case for the divergence of *columbiana* and *adusta* where they currently inhabit distinct ecological environments. Although *columbiana* and *flavapila* are sympatric at even a very local level, they represent distinct species. Therefore, the mechanism that promoted speciation between *flavapila* and its sister clade, *adusta-columbiana*, remains elusive. If
allopatric speciation played a role in their divergence, it is not evident from the current distribution of these taxa.

CONCLUSIONS

All datasets and analyses included in this research provided insight at various levels of investigation into the speciation of the four taxa of interest. These methods, when used synergistically, proved beyond a preponderance of evidence that the four morphospecies should be considered valid species. Multivariate morphological methods used here were insightful in proposing characters that could then be tested using a character-based analysis. Geometric morphometrics of wing vein shapes was helpful in not only identifying “hidden” characters, but will also be useful as a means of discriminating unknown individuals in the future through classification methods. DNA barcoding as well as nested clade phylogenetic analysis overestimated the number of species. However, gene trees provided a lot of insight into the potential mechanisms of speciation. Lastly, niche modeling differentiation methods indicated ecological speciation between two relatively recently diverged sister species.

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REFERENCES


LIST OF FIGURES

Figure 1. Map of *Neophylidorea* distribution. Points included are those for all specimens (museums and personal collection; indicated by a ring symbol) and those used in molecular analyses (indicated by a solid circle and labeled by an identifier). Morphospecies are marked by color: green = *columbiana*, blue = *adusta*, pink = *flavapila*, yellow = *caudifera*.

Figure 2. Landmarks digitized for geometric morphometrics analysis.

Figure 3. Hierarchical clustering using discrete morphological data. Each tip represents a single individual male specimen. All 19 characters are also displayed as bands of colors above each specimen and are numbered 1-19 corresponding to the characters listed in Table 2. The bands of colors furthest to the left indicate the morphospecies identity of each specimen. Green = *columbiana*, blue = *adusta*, pink = *flavapila*, yellow = *caudifera*.

Figure 4. Gene tree using mtDNA sequences analyzed through maximum likelihood analysis. Terminal taxa represent unique haplotypes and morphospecies identities are displayed using colored bars: green = *columbiana*, blue = *adusta*, pink = *flavapila*, yellow = *caudifera*. Bootstrap values >0.5 for major nodes are included and denote node support. Outgroup taxa are not shown for simplicity.

Figure 5. Gene tree using a fragment of the nuclear CAD gene analyzed using maximum likelihood analysis. Terminal taxa represent unique haplotypes and morphospecies identities are displayed using colored bars: green = *columbiana*, blue = *adusta*, pink = *flavapila*, yellow = *caudifera*. Bootstrap values >0.5 for major nodes are included and denote node support. Outgroup taxa are not shown for simplicity.

Figure 6. Haplotype network for clade 6-5 including all *adusta* taxa (blue) and two similar haplotypes of *columbiana* (green). Black circles indicate base pair changes linking haplotypes.
Several haplotypes are represented by multiple individuals, which are indicated by an ellipse and labeled with letters: A - A10109a, A3909a, A7309a, A9809a; B - A12309a, A12409a, A9909a; C - A7609a, A9209a.

**Figure 7. Haplotype network for clade 5-8 including all remaining *columbiana* (green)** haplotypes. Black circles indicate base pair changes linking haplotypes. Several haplotypes are represented by multiple individuals, which are indicated by an ellipse and labeled with letters: D - B0207a, B0207c; E - B1408a, B5707a; F - B4207b, B4407a.

**Figure 8. Haplotype networks for all *flavapila* (pink) taxa and the single network including both *caudifera* (yellow) specimens.** Black circles indicate base pair changes linking haplotypes. Several haplotypes are represented by multiple individuals, which are indicated by an ellipse and labeled with letters: G - T1608b, T1808a, T1808c; H - T2307c, T2307d; I - T5407b, T5407c; J - T5707a, T5707b; K - T5907a, T5907b.

**Figure 9. Principal component analysis of environmental data from background and known localities of *Neophylidorea* morphospecies.** Known localities are indicated by the following letters: A – *adusta* (blue), C – *columbiana* (green), D – *caudifera* (yellow), F – *flavapila* (pink). Background points are indicated by open circles with each morphospecies indicated using the same colors as known localities. All combinations of components 1, 2 and 3 are displayed.
Figure 1
Figure 3
Figure 5
Figure 8
Table 1. Locality information for each population collected in 2007-2009 and used for molecular sequencing. All specimens were adults, with the exception of a single individual from population T5407 (individual "c" as indicated in various analyses such as NCPA).

<table>
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<th>ID</th>
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<th>Locality</th>
<th>COI/COII</th>
<th>CAD</th>
<th>Latitude</th>
<th>Longitude</th>
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<td>42.5343</td>
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*Two individuals from this locality were males and therefore easily identifiable to morphospecies. One was identified as *columbiana* (B1708) the other as *flavapila* (T1708). Preliminary identification of the single female collected at the same locality was difficult but assumed to be *columbiana* based on wing shading and later confirmed through molecular sequencing.

Acronyms: CG = campground; Crk = creek; L = lake; Mnt = mountain; Mt = mount; NF = National Forest; NP = National Park; NWR = National Wildlife Refuge; PP = Provincial Park; Rd = road; Rv = river; SF = State Forest; SP = State Park; SRA = State Recreation Area; WMA = Wildlife Management Area.
Table 2. Characters and alternative character states used in the hierarchical clustering analysis.

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<th>Alternative character states</th>
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<td>Gonocoxite setae color</td>
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<td>9th tergite color</td>
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<td>Abdominal color</td>
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<td>Prescutum color</td>
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<td>9</td>
<td>Femur color*</td>
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<td>10</td>
<td>Scape color</td>
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<td>Flagellomere color</td>
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<td>12</td>
<td>Trichia on Rs</td>
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<tr>
<td>13</td>
<td>Cloud at wing cord</td>
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<tr>
<td>14</td>
<td>Cloud on R_{2,3,4}</td>
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<td>15</td>
<td>Apical wing clouding</td>
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<td>16</td>
<td>Cloud at base of Rs</td>
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<td>17</td>
<td>Spur on Rs</td>
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<td>18</td>
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<td>rounded</td>
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<tr>
<td>19</td>
<td>9th tergite median lobe shape</td>
<td>elongate</td>
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*if >50% of the femur is yellow, the color is considered “yellow” and likewise for brown.
Table 3. List of primers used including direction, whether the specific primer was used for amplification (PCR), sequencing or both. CAD sequences were modified from those used by Moulton and Wiegmann (2004).

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<th>Source</th>
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<td>X</td>
<td>GCTTGAGCTGGAATAATTGGCAC</td>
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<tr>
<td>TLN- 3017</td>
<td>Reverse</td>
<td>X</td>
<td>X</td>
<td>CTTAATCCATTGCACTAATCTGCC</td>
<td>Simon et al., 1994</td>
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<td>CAACATTATTTTGATTTTTTGG</td>
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<td>COII- 3037</td>
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<td>X</td>
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<td>X</td>
<td>GTTTAAGAGACCAGTACTTG</td>
<td>Simon et al., 1994</td>
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<td>CAD-Fc</td>
<td>Forward</td>
<td>X</td>
<td>X</td>
<td>GATTAYTCWGGHTCRCAAAGC</td>
<td>this study</td>
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<tr>
<td>CAD-Rc</td>
<td>Reverse</td>
<td>X</td>
<td>X</td>
<td>CGAACAATTTTYGCAATCC</td>
<td>this study</td>
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<td>Forward</td>
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<td>this study</td>
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<td>CAD-Ra</td>
<td>Reverse</td>
<td>X</td>
<td>X</td>
<td>ACYTCATAYTCRACYTCYTTC</td>
<td>this study</td>
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</table>
Table 4. Results from assessment based on Wiens and Servedio (2000) identifying diagnostic
differences between morphospecies based on 19 morphological characters (c) and using a frequency
cut-off of 0.10. A significant result indicates acceptance of the hypothesis that an alternative
character state of those seemingly fixed characters would be <10% likely in the population as a
whole.

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<th>n (B)</th>
<th>k (characters)</th>
<th>p-value (A, B)</th>
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<td>90</td>
<td>3 (15, 18, 19)</td>
<td>&lt;0.05*, &lt;0.01**</td>
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<td>adusta-caudifera</td>
<td>69</td>
<td>17</td>
<td>3 (16, 18, 19)</td>
<td>&lt;0.05**, NS</td>
</tr>
<tr>
<td>flavapila-columbiana</td>
<td>90</td>
<td>81</td>
<td>2 (18, 19)</td>
<td>&lt;0.01**, &lt;0.01**</td>
</tr>
<tr>
<td>flavapila-caudifera</td>
<td>90</td>
<td>17</td>
<td>2 (18, 19)</td>
<td>&lt;0.01**, NS</td>
</tr>
<tr>
<td>columbiana-caudifera</td>
<td>81</td>
<td>17</td>
<td>3 (6, 18, 19)</td>
<td>&lt;0.01**, NS</td>
</tr>
</tbody>
</table>
Table 5. Discriminate function analysis results for each morphospecies including percentage of individuals discriminated to the respective morphospecies and of those incorrect discriminations, which morphospecies was indicated by the analysis. Average relative warp (RW) scores for RW1 and RW2 are included along with a distance-based measure of disparity, 95% confidence interval calculated from 100 bootstrap replicates and standard error of disparity.

<table>
<thead>
<tr>
<th>Morphospecies</th>
<th>N</th>
<th>% correctly discriminated</th>
<th>Incorrect discriminations</th>
<th>Average coordinates (RW1, RW2)</th>
<th>Distance-based disparity</th>
<th>95% confidence interval</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>adusta</td>
<td>151</td>
<td>88.7</td>
<td>11-caudifera</td>
<td>-0.0062, 0.0179</td>
<td>0.00495</td>
<td>0.00457, 0.00526</td>
<td>0.00018</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-columbiana</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2-flavapila</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caudifera</td>
<td>24</td>
<td>87.5</td>
<td>3-adusta</td>
<td>0.0302, 0.0219</td>
<td>0.00434</td>
<td>0.00320, 0.00494</td>
<td>0.00047</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8-adusta</td>
<td>0.0199, -0.0033</td>
<td>0.00495</td>
<td>0.00447, 0.00547</td>
<td>0.00025</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3-flavapila</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-caudifera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>columbiana</td>
<td>124</td>
<td>90.3</td>
<td>3-columbiana</td>
<td>-0.0208, -0.0264</td>
<td>0.00656</td>
<td>0.00599, 0.00711</td>
<td>0.00028</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-adusta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-caudifera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flavapila</td>
<td>107</td>
<td>95.3</td>
<td>3-columbiana</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-adusta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-caudifera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Significant inferences derived from NCPA using the chain of inference developed by Templeton (1998) to assess genetic associations with geography. Dc is the clade distance between the interior and tip clades. Dn is the nested clade distance between the interior and tip clades.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Inference</th>
<th>Inference</th>
<th>Dc (I-T)</th>
<th>Dn (I-T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4</td>
<td>1-2-11-17 NO</td>
<td>Inconclusive</td>
<td>379.885</td>
<td>-41.075</td>
</tr>
<tr>
<td>3-11</td>
<td>1-19 NO</td>
<td>Allopatric fragmentation</td>
<td>188.264*L</td>
<td>140.720</td>
</tr>
<tr>
<td>3-12</td>
<td>1-19 NO</td>
<td>Allopatric fragmentation</td>
<td>-4.905</td>
<td>340.705*L</td>
</tr>
<tr>
<td>4-3</td>
<td>1-19-20-2 IO</td>
<td>Inconclusive</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5-2</td>
<td>1-2-3-4 NO</td>
<td>Restricted gene flow with isolation by distance</td>
<td>241.5188</td>
<td>184.305</td>
</tr>
<tr>
<td>5-8</td>
<td>1-2 IO</td>
<td>Inconclusive</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6-5</td>
<td>1-19 NO</td>
<td>Allopatric fragmentation</td>
<td>1425.629*L</td>
<td>1423.856*L</td>
</tr>
<tr>
<td>7-5*</td>
<td>1-19-20-2 IO</td>
<td>Inconclusive</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Total</td>
<td>1-2 IO</td>
<td>Inconclusive</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

*Clade 7-5 is not pictured but includes the subclades 6-5 and 5-8.

All others the null hypothesis could not be rejected.
Table 7. PCA loading scores and percentage of variance explained by each principal component.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td>36.4</td>
<td>32.8</td>
<td>10.9</td>
<td>7.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Cumulative Percent</td>
<td>36.3</td>
<td>69.1</td>
<td>80.0</td>
<td>87.6</td>
<td>92.6</td>
</tr>
<tr>
<td>Compound Topographic Index</td>
<td>-0.0048</td>
<td>-0.0009</td>
<td>0.0297</td>
<td>0.0159</td>
<td>0.9889</td>
</tr>
<tr>
<td>Annual Mean Temperature</td>
<td>0.2862</td>
<td>0.2051</td>
<td>0.2227</td>
<td>-0.0659</td>
<td>0.0076</td>
</tr>
<tr>
<td>Mean Diurnal Range</td>
<td>0.3175</td>
<td>-0.0589</td>
<td>-0.0216</td>
<td>-0.0520</td>
<td>-0.0857</td>
</tr>
<tr>
<td>Isothermality</td>
<td>0.2911</td>
<td>0.1826</td>
<td>-0.1639</td>
<td>0.0527</td>
<td>-0.0181</td>
</tr>
<tr>
<td>Temperature Seasonality</td>
<td>-0.1349</td>
<td>-0.3199</td>
<td>0.2463</td>
<td>0.0055</td>
<td>-0.0313</td>
</tr>
<tr>
<td>Max Temperature of Warmest Month</td>
<td>0.3209</td>
<td>0.0447</td>
<td>0.2682</td>
<td>-0.1032</td>
<td>-0.0387</td>
</tr>
<tr>
<td>Min Temperature of Coldest Month</td>
<td>0.2271</td>
<td>0.3011</td>
<td>0.0098</td>
<td>-0.0439</td>
<td>0.0295</td>
</tr>
<tr>
<td>Temperature Annual Range</td>
<td>-0.0152</td>
<td>-0.3435</td>
<td>0.2154</td>
<td>-0.0321</td>
<td>-0.0702</td>
</tr>
<tr>
<td>Mean Temperature of Wettest Quarter</td>
<td>0.1471</td>
<td>-0.0863</td>
<td>0.5322</td>
<td>0.0950</td>
<td>0.0252</td>
</tr>
<tr>
<td>Mean Temperature of Driest Quarter</td>
<td>0.2029</td>
<td>0.2676</td>
<td>-0.1839</td>
<td>-0.0934</td>
<td>-0.0094</td>
</tr>
<tr>
<td>Mean Temperature of Warmest Quarter</td>
<td>0.2819</td>
<td>0.0792</td>
<td>0.3850</td>
<td>-0.0836</td>
<td>-0.0092</td>
</tr>
<tr>
<td>Mean Temperature of Coldest Quarter</td>
<td>0.2609</td>
<td>0.2720</td>
<td>0.0477</td>
<td>-0.0467</td>
<td>0.0176</td>
</tr>
<tr>
<td>Annual Precipitation</td>
<td>-0.2280</td>
<td>0.2887</td>
<td>0.1451</td>
<td>0.0645</td>
<td>-0.0300</td>
</tr>
<tr>
<td>Precipitation of Wettest Month</td>
<td>-0.1849</td>
<td>0.2746</td>
<td>0.1255</td>
<td>0.3565</td>
<td>-0.0358</td>
</tr>
<tr>
<td>Precipitation of Driest Month</td>
<td>-0.2390</td>
<td>0.2230</td>
<td>0.1374</td>
<td>-0.3420</td>
<td>-0.0006</td>
</tr>
<tr>
<td>Precipitation Seasonality</td>
<td>0.1614</td>
<td>-0.0663</td>
<td>0.0619</td>
<td>0.6776</td>
<td>0.0047</td>
</tr>
<tr>
<td>Precipitation of Wettest Quarter</td>
<td>-0.1923</td>
<td>0.2749</td>
<td>0.1212</td>
<td>0.3367</td>
<td>-0.0367</td>
</tr>
<tr>
<td>Precipitation of Driest Quarter</td>
<td>-0.2418</td>
<td>0.2339</td>
<td>0.1321</td>
<td>-0.3193</td>
<td>-0.0077</td>
</tr>
<tr>
<td>Precipitation of Warmest Quarter</td>
<td>-0.2422</td>
<td>0.0761</td>
<td>0.4206</td>
<td>-0.0288</td>
<td>-0.0103</td>
</tr>
<tr>
<td>Precipitation of Coldest Quarter</td>
<td>-0.1604</td>
<td>0.3161</td>
<td>-0.0841</td>
<td>0.1609</td>
<td>-0.0364</td>
</tr>
</tbody>
</table>
Table 8. Results of niche identity and background similarity tests. Niche identity tests are one-tailed while background tests are two-tailed. Significant niche identity tests indicate the measured \( I \) is for any species pair is less similar than predicted based on bootstrap values. Actual identity is more or less similar than expected.

<table>
<thead>
<tr>
<th>Morphospecies A - B</th>
<th>( I )</th>
<th>Identity test</th>
<th>A versus B background</th>
<th>B versus A background</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adusta-columbiana</em></td>
<td>0.379</td>
<td>P&lt;0.01</td>
<td>Less (P&lt;0.05)</td>
<td>NS</td>
</tr>
<tr>
<td><em>adusta-flavapila</em></td>
<td>0.392</td>
<td>P&lt;0.01</td>
<td>Less (P&lt;0.01)</td>
<td>Less (P&lt;0.05)</td>
</tr>
<tr>
<td><em>adusta-caudifera</em></td>
<td>0.733</td>
<td>P=0.05</td>
<td>More (P&lt;0.01)</td>
<td>NS</td>
</tr>
<tr>
<td><em>flavapila-columbiana</em></td>
<td>0.731</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td>More (P&lt;0.01)</td>
</tr>
<tr>
<td><em>flavapila-caudifera</em></td>
<td>0.491</td>
<td>P&lt;0.01</td>
<td>More (P&lt;0.01)</td>
<td>Less (P&lt;0.01)</td>
</tr>
<tr>
<td><em>columbiana-caudifera</em></td>
<td>0.424</td>
<td>P&lt;0.01</td>
<td>More (P&lt;0.05)</td>
<td>Less (P&lt;0.01)</td>
</tr>
</tbody>
</table>
Table 9. Summary of results from all datasets and methods used to test morphospecies hypotheses.

<table>
<thead>
<tr>
<th>Morphospecies</th>
<th>Morphology</th>
<th>Geometric Morphometrics</th>
<th>DNA</th>
<th>Ecological Niche</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tree-based</td>
<td>Character-based DFA MANOVA</td>
<td>Tree-based mtDNA</td>
<td>Tree-based nDNA</td>
</tr>
<tr>
<td>adusta</td>
<td>distinct</td>
<td>indistinct distinct</td>
<td>non-exclusive with cambium non-exclusive with adusta distinct</td>
<td>non-exclusive with cambium non-exclusive with adusta distinct</td>
</tr>
<tr>
<td>colombiana</td>
<td>distinct</td>
<td>distinct distinct distinct</td>
<td>distinct from cambium distinct from cambium distinct</td>
<td>distinct from cambium distinct from cambium distinct</td>
</tr>
<tr>
<td>flavapila</td>
<td>distinct</td>
<td>distinct distinct distinct</td>
<td>distinct from cambium distinct from cambium distinct</td>
<td>distinct from cambium distinct from cambium distinct</td>
</tr>
<tr>
<td>caudifera</td>
<td>distinct</td>
<td>insufficient data distinct</td>
<td>distinct from adusta insufficient data distinct</td>
<td>insufficient data insufficient data distinct</td>
</tr>
</tbody>
</table>
APPENDIX 1

*Neophylidorea* taxonomy currently consists of 16 described species. Fourteen of those species are considered in the current work delimiting species. The remaining two species are excluded due to small sample sizes of specimens, but the available specimens suggest they are morphologically distinct. Described below are the described species that group together based on male genitalia and geography and are throughout the text referred to as morphospecies.

**adusta**
1. *N. adusta* (Osten Sacken 1859)

**caudifera**
1. *N. caudifera* (Alexander 1927)

**columbiana**
1. *N. columbiana* (Alexander 1927)
2. *N. snoqualmiensis* (Alexander 1945)
3. *N. pacalis* (Alexander 1949)
5. *N. brevifilosa* (Alexander 1959)
6. *N. burdicki* (Alexander 1964)
7. *N. olympica* (Alexander 1949)

**flavapila**
1. *N. flavapila* (Doane 1900)
2. *N. aleutica* (Alexander 1920)
3. *N. tepida* (Alexander 1926)
Hierarchical clustering based on male and female data. 416 individuals were analyzed using Ward’s linkage. Branch lengths are proportional to distances between individuals at the terminals.

The far left column is colored based on the morphospecies identity of the individual: green = *columbiana*, blue = *adusta*, pink = *flavapila*, yellow = *caudiera*. 
APPENDIX 3

Principal component analysis on the covariance of the weight matrix (partial warp scores). The first axis explained 20.19% and the second axis explained an additional 15.63% of the total variation. The stars represent the morphospecies means. Polygons represent the minimum convex hulls for each morphospecies. Green = *columbiana*, blue = *adusta*, pink = *flavapila*, yellow = *caudifera*. 
APPENDIX 4

There was no "barcoding gap" in this dataset using uncorrected pair-wise distances. In other words, no maximum intraspecific distance can be used to determine species limits.

The following table displays the mean uncorrected pair-wise interspecific and intraspecific (along the diagonal in bold including the minimum, mean ± standard error, and maximum) distances as percentages of the total number of base pairs (n=1411) with associated standard errors. Morphospecies *caudifera* was just represented by two individuals so there is just a single intraspecific comparison and therefore no minimum, standard error or maximum.

<table>
<thead>
<tr>
<th>Morphospecies</th>
<th><em>adusta</em></th>
<th><em>columbiana</em></th>
<th><em>caudifera</em></th>
<th><em>flavapila</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>adusta</em></td>
<td>0, 0.3 ± 0.0, 1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>columbiana</em></td>
<td>2.3 ± 0.3</td>
<td>0, 1.3 ± 0.2, 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>caudifera</em></td>
<td>8.5 ± 0.8</td>
<td>7.7 ± 0.7</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><em>flavapila</em></td>
<td>6.7±0.6</td>
<td>6.0 ± 0.6</td>
<td>8.1 ± 0.7</td>
<td>0, 1.9 ± 0.2, 5.6</td>
</tr>
</tbody>
</table>
Three additional barcoding criteria were used to detect species limits. The "best close match" was determined using a threshold of 6.01% divergence based on the 95th percentile all pair-wise comparisons.

<table>
<thead>
<tr>
<th>Identification Criteria</th>
<th>Correct</th>
<th>Ambiguous</th>
<th>Incorrect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best Match</td>
<td>76 (97.43%)</td>
<td>0 (0.0%)</td>
<td>2 (2.56%)</td>
</tr>
<tr>
<td>Best Close Match</td>
<td>76 (97.43%)</td>
<td>0 (0.0%)</td>
<td>2 (2.56%)</td>
</tr>
<tr>
<td>All Species Barcodes</td>
<td>11 (14.1%)</td>
<td>67 (85.9%)</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

The two individuals that were incorrectly identified using “best match” were the same as those misidentified through “best close match”. A9509a (adusta) incorrectly matched B1708a (columbiana) and T1708a (flavapila) incorrectly matched B1708b (columbiana). The second match is not necessary incorrect. The individual, B1708b was a female collected at the same locality as T1708a, an identified species based male based on genitalia. The female, B1708b was only circularly identified as colombiana because in both gene trees (Figures 4 and 5) it is sister to a male specimen identified as colombiana based on genitalia, B1708a, and only separated by a single basepair change (Figure 6). The only individuals that were correctly identified using the “all species barcode” criteria were a subset of flavapila individuals.
APPENDIX 5

The following figures represent all pair-wise morphospecies comparisons depicting niche identity and background permutation test results. The arrow represents the calculated value of $I$, or measured niche overlap where zero is completely unique and one is identical. The histograms represent the distribution of overlap from pseudoreplicates for the various tests. Three tests are presented: 1. Niche identity, 2. Background niche identity for species A compared to the background of species B, and 3. Background niche identity for species B compared to the background of A. For each test, 100 total replicates were performed and is displayed as a frequency distribution.
adusta versus caudifera
background
caudifera versus adusta
background
identity
columbiana versus
flavapila background
flavapila versus
columbiana background
identity
caudifera versus flavapila background
flavapila versus caudifera background
identity

columbiana versus caudifera background
caudifera versus columbiana background
identity
CHAPTER FOUR: SYSTEMATIC REVISION AND PHYLOGENETICS OF *NEOPHYLIDOREA*  
(DIPTERA: TIPULOIDEA)  

A paper to be submitted to *Systematic Entomology*

Abstract

Hypothesis driven revisionary taxonomy is presented here as a thorough investigation of the morphology and phylogenetic relationships of the six species comprising the genus *Neophylidorea* Petersen and Courtney. Through this revisionary analysis, ten formerly valid species are synonymized. One additional species is considered invalid due to lack of distinguishing characters in female morphology and an inability to associate it with any previously described species. This monophyletic genus has an entirely Nearctic distribution, which has resulted in broad sampling and relatively large holdings in existing museum collections. Because of the large number of specimens examined during the course of this revision, clines between previously described species were observed. Six valid species, four that have been previously quantitatively delimited, are described in detail based on many new combinations. One of the six is a new species, *Neophylidorea vanronea*, described based on two male specimens collected in Michigan, USA. This species can be distinguished by the unique structure of the ventral parameres in the male hypopygium. The phylogenetic relationships among species are hypothesized using a combination of molecular and morphological data.

**Keywords**: Tipuloidea, Limnophilinae, morphology, COI, COII, CAD

Introduction

The goal of systematic biology is to document diversity and understand the phylogenetic relationships among species. Of particular importance is to describe the biodiversity on Earth and the
conservation potential of species during the current biodiversity crisis (Wilson 2000, Dubois 2003). Unfortunately, some continue to view taxonomy as a service to the greater scientific community and a science that lacks hypothesis testing (Wheeler 2004). However, the process of describing a new species is equivalent to proposing a hypothesis. Hypotheses in any aspect of science have the potential to be disproven. The same is true for taxonomic hypotheses where errors in species hypotheses may inflate or deflate (e.g., undetected cryptic species) the true known diversity (May and Nee 1995). To best understand the diversity of life, there is a need for systematic biology and taxonomic revisions specifically.

Crane flies are incredibly diverse, representing nearly 10% of all described species of Diptera (>15,000 species of crane flies worldwide; de Jong et al. 2008, Oosterbroek 2010). Many species have been described and the majority (11,278) by a single author, Charles Alexander (Oosterbroek 2009). However, crane fly taxonomy has suffered greatly from a lack of comprehensive revisionary work and, therefore, the diversity may be over estimated. Few revisions are comprehensive, or include all members of a monophyletic group (e.g., Brodo 1987, de Jong 1989, Gelhaus 2005, Petersen 2008). Many other revisions are regional, or cover only a portion of the taxa and may not represent monophyletic groups, making phylogenetic conclusions difficult (e.g., Byers 1961, Starý 1987, Young 1987, Starý 2003).

Disciplines such as biodiversity research, ecology, and conservation biology, species are the fundamental unit. From a theoretical perspective, others may disagree and argue that species “have no special reality in nature” (Mishler and Donoghue 1982). A great deal of attention has been paid to concepts or methodology used to define the limits of species. One goal of defining species as the fundamental unit of biology is to create a hierarchical level that is equivalent across taxa. One problem with this goal is that taxa may differ in the methods necessary to define species limits. The species concept used here is that of independently evolving metapopulation lineages (de Queiroz 2005). This is the preferred method because it allows for a variety of methods to delimit species. Multivariate operational methods incorporating ecology, morphology, and genetic data were used to
delimit species of the genus Neophylidorea Petersen and Courtney 2010 (de Queiroz 2005; Chapter 3).

**Taxonomic History**

Prior to this revision, systematists have paid little attention to the species that under the current taxonomy are considered Neophylidorea. At the turn of the century two independent entomologists, Rennie Wilbur Doane and Baron Carl Robert Osten Sacken each described one of the six species included in the present revision. All remaining species were described in isolated publications by Charles Alexander from 1916-1964. Most of these early species were described as members of the genus Limnophila Macquart or L. (Phylidorea) Bigot. In 1972, Alexander described a new genus, Euphylidorea, into which he placed most species considered here.

The original descriptions provided little morphological information that could be used to identify species. Comprehensive taxonomic keys were also lacking prior to this revision. Likewise, biological or distributional information was limited to short comments such as “shaded moist places” or “along streams and in woodlands” (Alexander 1942).

Chapter 2 describes the new genus Neophylidorea, which includes 16 species that were previously considered Euphylidorea. Characters of the aedeagus and male genitalia in general allow for unequivocal recognition of this genus. Specific synapomorphies of Neophylidorea include a long, basally trifid aedeagus with the median aedeagal filament curving dorsally away from the lateral filaments just apical to the split from the short common stem before straightening out, prominent median process on the ninth tergite of variable shape, aedeagal filaments are entire unlike other species of Euphylidorea, and have a relatively short radial sector vein. The presence of a basally trifid aedeagus is an autapomorphic character within the “Limnophilinae”.

The work presented here takes a broad approach to taxonomy by reviewing the biology of each Neophylidorea species and the data that support each species hypothesis. The objectives of this research are to adequately review and illustrate the morphological limits of each species, describe the known biology, provide a dichotomous key to adults, identify specimens that are
otherwise unidentifiable to species through discriminate function analysis, estimate the potential geographic distribution of species and propose the evolutionary relationships among species of *Neophyldorea* through parsimony analysis of morphological data and Bayesian analysis of molecular and combined data.

**Materials and Methods**

**Taxonomy**

Approximately 500 specimens from a variety of museums were studied to understand the morphology and phylogenetic relationships of *Neophyldorea* species. Museums included: Academy of Natural Sciences, Philadelphia, Pennsylvania (ANSP, J. Gellhaus); California Academy of Sciences, San Francisco, California (CAS, N. Penny); Canadian National Collection, Ottawa, Canada (CNC, J.M. Cumming); Carnegie Museum of Natural History (C. Young); Cornell University Insect Collection (J. Liebherr); personal collection of Fenja Brodo, Ottawa, Canada (FB); Iowa State Insect Collection, Ames, Iowa (ISIC); Museum of Comparative Zoology, Cambridge, Massachusetts (MCZ, P. Perkins); University of Michigan Museum of Zoology, Ann Arbor, Michigan (UMMZ, M.F. O’Brien); and collections of the United States National Museum, deposited in the National Museum of Natural History (NMNH), Smithsonian Institution, Washington, D.C. (USNM, W.N. Mathis).

The majority of museum specimens were adhered to points of cardstock (“pointed”), unless noted otherwise. Male genitalia were preserved in glycerin in a microvial or slide mounted in Canada balsam. Additional collecting trips were made in 2007, 2008 and 2009 to study the biology, better understand the geographic distribution and collect fresh specimens. Attempts were made to collect from type localities, but this often proved difficult due to apparent habitat alterations, incomplete descriptions of holotype locations or likely shifts in microhabitat phenology causing earlier or later emergence dates. Specimens were generally swept from vegetation and direct killed into 95% ethyl alcohol (EtOH), although some individuals were collected into ethyl acetate, stored in glassine envelopes and pointed as vouchers. Night collecting or black-lighting generally proved difficult due to low nighttime temperatures during the flight period.
Genitalic features were examined by clearing terminalia in warm potassium hydroxide (KOH) solution for 10-20 minutes, rinsing in distilled water, transfer to 35% ethanol, then moved up through a series of increasing concentrations of ethanol every hour to a final concentration of 90%. Terminalia were then transferred to a solution of 1:1 ethanol to glycerin and incubated at room temperature while the ethanol evaporated. Terminalia were then transferred to fresh glycerin and stored in microvials attached to the remainder of the pinned specimen. Coloration is often an important character used in crane fly taxonomy. Factors that can change the color include glue, adhesive, specimen age, method of preservation (slide, pinned, pointed, alcohol), presence of eggs in the abdomen of females, specimen maturity (e.g., teneral individuals are lighter), and light type (daylight, microscope, fluorescent). When making coloration judgments, the specimen was viewed through a dissecting microscope and these various factors were taken into consideration.

The characters and terminology follow McAlpine (1981) and Alexander and Byers (1986). Worth noting are some of the male genitalic terminology used by Alexander and the corresponding terminology used here. Inner and outer dististyles are here described more specifically as the dorsal and ventral gonostylus respectively. The basistylose is described as the gonocoxite here. Alexander often referred to the lateral filaments of the aedeagus as the subtending gonapophyses (1927a, 1927b), subtending apophyses (1927a), or lateral filaments (1964). Ventral parameres were often referred to as simple (Alexander 1927a, 1959, 1961) or basal (Alexander 1958) gonopophyses or even apophyses, causing some confusion with the branches of the aedeagus (Alexander 1926).

Measurements were taken using a dissecting microscope and an eyepiece equipped with a scale bar to the nearest 0.5 mm. Body length was measured from the tip of the rostrum to the apex of the abdomen. Where material was misshapen, individual sections (head, thorax, abdomen) were measured and summed. Wing length was measured from the thoracic connection to the wing apex. Reported length indicates the means of all specimens with the observed ranges and sample sizes in parentheses.

Illustrations were produced from cleared terminalia in glycerin or in some cases slide mounted terminalia in Canada balsam (e.g., *N. neadusta* and *N. vanrorea* sp. nov.). Most
illustrations were rendered using a camera lucida connected to a Nikon dissecting microscope, converted to a digital format and drawn using Macromedia Flash.

For each treatment of a taxonomic entity, the original reference to the taxon is given along with any previous or newly synonymized taxa. The species are listed in phyletic sequence as determined by the phylogenetic analysis presented below. For each species a diagnosis is made, followed by a description of the adult male and female. Most aspects of the general morphology do not vary between species including descriptions of the head and thorax. For a general description of the genus, see Chapter 2. Unless noted otherwise, the species-level morphology is as stated in the generic description. Where available, immature life stages are also described or referenced. The material examined is listed, including type material, non-type material, locality information, deposition location, and associated slide mountings or other remarks (e.g., multiple specimens pinned together).

The geographic distribution of each species was estimated using ecological niche modeling (Kozak et al. 2008). This method has been shown to provide accurate estimates of potential distribution by combining coarse grain environmental data with known localities of the taxa of interest (Peterson 2001; Elith et al. 2006). Ecological niche modeling was performed using Maximum entropy modeling through the program Maxent version 3.3.1 (Phillips et al. 2004; Phillips et al. 2006; Phillips and Dudík 2008). Maxent was chosen here because in comparative analyses it has consistently outperformed other modeling algorithms (Elith et al. 2006; Hernandez et al. 2006). This method uses iterative correlations between presence-only occurrences and environmental layers to develop a model of potential distribution. Occurrence data was derived from museum specimens and collections made by the author in 2007-2009. Environmental variables included 19 bioclimatic variables from the Worldclim dataset (Hijmans et al. 2005) and Compound Topographic Index at a spatial resolution of 2.5 arc-seconds (Table 1). Jackknifing was used to eliminate layers prone to overfitting the occurrence points (DeVaney et al. 2009). Twenty models were developed from occurrence data randomly divided into 50:50 training and testing datasets. Correlation coefficients were calculated between inclusion of each environmental variable and omission error in the test datasets. Environmental layers with a positive correlation with omission error (r>0.1) were removed.
Jackknifing was repeated until no strongly positive correlations with omission error remained. Final models were averaged over 20 runs, 25% test data removed from model training for subsequent testing and the ‘fade-by-clamping’ was used to remove regions outside the environmental limits of the model building. Model evaluation was based on area under the curve (AUC) and proportion of test occurrences omitted from a binomial model using a minimum training presence threshold. The resulting model and locations of specimens were mapped for each species. Species known from only single locality (N. vanronea sp. nov. and N. neadusta) were excluded from this analysis.

The phenology is discussed with regards to number of generations, length of flight period, and correlation between Julian date and latitude. We hypothesize that earlier emerging populations (smaller Julian dates) occur at lower latitudes (i.e., a positive relationship between Julian date and latitude). This hypothesis is tested with each species where enough data are available (N. adusta, N. caudifera, N. columbiana, and N. flavapila) using regression in the program JMP version 8. Biology is discussed regarding associated crane fly species and closely related groups (e.g., Ptychopteridae) noted during the time of collection, the dominant plant community and the habitat type. Water temperature and pH of adjacent habitats was recorded at the time of collection. Water temperature and pH were measured at 3” below the surface. Lastly, remarks on synonymy, where appropriate are discussed.

Some female specimens or specimens lacking genitalia were examined, but lacked species defining characters. The species determination was predicted using the formula created from a discriminate function analysis (DFA) of wing geometric morphometrics built from known species identities (Chapter 3). The probability cut-off for species determinations using DFA is 0.95, based on the level of accurate predictions from the test dataset (Chapter 3). Analyses were performed in JMP version 8.

Phylogenetics

Molecular and morphological data were used to analyze the evolutionary relationships within and among species of Neophyliidorea. Three genes, two mitochondrial and one nuclear, from 23
individuals including three outgroup taxa were sequenced and analyzed (Table 2). Two mitochondrial genes were sequenced, including cytochrome c oxidase subunits I and II (COI and COII). These genes are relatively fast evolving and are commonly used for species-level phylogenetics (Simon et al. 1994; Lunt et al. 1996). A portion of the coding nuclear gene carbamoyl phosphate synthase domain of the rudimentary locus (CAD) was also sequenced. This gene has been commonly used in Diptera systematics at various hierarchical levels of investigation (Moulton and Wiegmann 2004, Petersen et al. 2010).

Methods involving DNA extraction, PCR amplification, primer selection and sequence alignment are explained in greater detail elsewhere (Chapter 3). In general, whole DNA was extracted from the entire body of individuals using the DNeasy Tissue Kit (Qiagen) according to the manufacture’s protocols. PCR was run in 10 µl amplification reactions. Each reaction consisted of 1 µl DNA, 10 µl Tris-HCl (pH 8.0), 50 µl KCl, primers, and 0.025 U of Taq Jumpstart polymerase (Sigma). Thermal cycling profiles in general consisted of a denaturing step at 95°C for 5 min; 32-35 cycles of denaturing at 95°C for 60 s, annealing at 58°C (COI, COII) or 48°C (CAD) for 60 s, and extension at 72°C for 2 min; and a final extension at 72°C for 5 min.

Primers and unincorporated nucleotides were digested by adding 0.5 U Exonuclease (USB) and 0.5 U Shrimp Alkaline Phosphatase (USB) to 8 µl of PCR product. Samples were incubated for 30 min at 37°C, then 10 min at 90°C. Cycle sequencing was conducted using BigDye 3.1 (Applied Biosystems) with the recommended cycling conditions. Sequences were read using Applied Biosystems model 3730 automated DNA sequencers. Most fragments were confirmed by sequencing both the forward and reverse strands. Sequences were checked, concatenated (COI and COII) and alignments were made using the program Sequencher 4.5 (Genecodes). Sequences were subjected to BLAST analysis and checked for pseudogenes by looking for any premature stop codons.

Bayesian analyses were performed with MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) for each molecular dataset (mitochondrial and nuclear) separately as well as a combined partitioned dataset. Akaike information criterion (AIC) was used to
select an evolutionary model that best fits the observed data using the program jModeltest (Posada 2008). The general time-reversible gamma-shape parameter and proportion of invariant sites to estimate rate heterogeneity (GTR+G+I) model was selected and site-specific rates were estimated for each data type (mitochondrial and nuclear). Two simultaneous runs of four Markov Chain Monte Carlo chains were allowed to search for $10^6$ generations for adequate time of convergence. Trees were sampled every 100 generations with 2500 discarded as initial burn-in. Final trees were compiled into a 50% majority-rule consensus tree with associated clade credibility indicated by posterior probabilities.

Morphological character states were derived from the same specimens used in molecular analyses, with a few exceptions. Specimens of *N. neadusta* and *N. vanronea* sp. nov. were not collected during the course of this study. Therefore, molecular data for these species is lacking and morphological character states were derived from the holotype specimens.

The program TNT was used to analyze combined morphological and molecular data (Goloboff et al., 2003, Goloboff et al. 2008). The “Traditional” search function was employed with a total of 1,000 random sequences using TBR branch swapping and holding 50,000 trees. The most parsimonious trees (MPT) found were saved and a strict consensus topology was calculated using the “Nelsen” option in TNT. Support was evaluated by 1,000 bootstrap (bs) replicates employing TBR.

**Results**

**Taxonomy**

Six species of *Neophylidorea* are included as valid species in this revision. Five species (*N. adusta, N. flavapila, N. caudifera, N. columbiana*, and *N. neadusta*) represent new combinations based on analytical methods of delimiting species (Chapter 3). *Neophylidorea vanronea* is described as a new species. Two other species (*N. olympica* and *N. aequiatra*) described by Alexander are based only on female holotypes and are considered invalid species. Two methods, geometric morphometrics could have been used to connect these holotypes to valid species of *Neophylidorea*, but the resources were unavailable. The wings of the holotypes of *N. olympica* and *N. aequiatra* were
not flat enough to be included in a discriminate function analysis of wing shape. Likewise, we were unable to collect fresh specimens for molecular analysis from the holotype locations.

Species of *Neophylidorea* include:

1. *N. caudifera* (Alexander 1927) [new combination]
2. *N. flavapila* (Doane 1900) [new combination]
3. *N. vanronea* [new species]
4. *N. neadusta* (Alexander 1927) [new combination]
5. *N. columbiana* (Alexander 1927) [new combination]
6. *N. adusta* (Osten Sacken 1859) [new combination]

*Neophylidorea caudifera* (Alexander, 1927)

*Limnophila (Phylidorea) caudifera* Alexander, 1927a: 111 [original designation].

**DIAGNOSIS.** The morphology of *N. caudifera* is unique, including the following characters: ventral paramere elongate with rounded apex, aedeagal filaments stout and shorter than other species in the genus, central aedeagal filament bends dorsally as in other species but to a lesser degree, and median lobe of the ninth tergite elongate and pronounced with dorsal processes nearly absent.

**DESCRIPTION.** Adult. MALE (Figure 1). **Measurements** (N=18): body length: 8.72 mm (7-10 mm); wing length: 9.19 mm (8-10 mm). **Legs.** Brown band on distal 1/8th of femur, remainder yellow. **Wing** (Figure 2). **Rs** short. **Abdomen.** Yellow, terminal darkened ring on 8th segment. **Hypopygium.** Thick, long, yellow setae on dorsal portion of gonocoxite pointed medially (Figure 1A, E); patch of short, thick, brown setae on the inner surface of the gonocoxite (Figure 1A); outer gonostylus wide; gonostylus glabrous or nearly so. FEMALE. **Measurements** (N=8): body length: 10.44 mm (9-11.5 mm); wing length: 10.13 mm (9-11.5 mm). **Pupa.** Undescribed. **Larva.** Undescribed.

**TYPE MATERIAL.** Holotype. New York, Lake Pleasant, 1750 ft., 1 M, 17 VI 1926, Alexander (USNM); Paratype. As in holotype, but 1 M, 21 VI 1926 [slide mounted wing, genitalia, remainder pointed].
OTHER MATERIAL EXAMINED (N=31). CANADA: Manitoba: Aweme, N. Crindle, 1 M, 20 V 1922, Unknown (CNC); as in preceding but 1 F, 3 VI 1920; Ontario: Oak Ridges Moraine, limnocrene spring, 1 M, 1 VI 1996, Gathmann (CNC) [specimen in alcohol, collected from emergence trap] (Gathmann and Williams 2006); Saskatchewan: Saskatoon, 1 M, 3 VI 1948, Vockeroth (CNC).

UNITED STATES: Maine: Augusta, 1 M, 22 V 1941, Brower (USNM) [slide mounted genitalia, wing, leg, antenna, remainder pointed]; Michigan: Hell Creek, Livingston Co, 3 MM, 21 V 1950, Rogers (UMMZ); Iosco Co. 2 MM, 1 F, 3 VI 1948, Rogers (UMMZ) [1 M study specimen #3916 entire specimen slide mounted]; Livingston Co., 1 M, 1 F, 31 V 1947, Rogers (UMMZ); as in preceding but 5 MM, 1 F, 21 V 1950; Lossing-Harrington Preserve, N45° 26.307' W84° 47.505', 640 ft., 3 MM, 29 V 2009, J. Petersen (ISIC) [95% EtOH]; W. Branch Sturgeon River, 1 M, 27 V 1948, Leonards (UMMZ); Washtenaco Co., 1 M, 4 VI 1931, Rogers (UMMZ) [study specimen #1166, entire specimen slide mounted]; New York: Indian Lake, N43.7583° W74.1705°, 1 M, 6 VI 1980, McCabe (ANSP); South Dakota: Harney Trail, Harney National Forest [Black Hills], 1 M, 1 F, 12 VI 1950, Byers (KSU) [study specimen #5513, slide mounted genitalia, wing, remainder pointed]; Vermont: Manchester, Bennington Co., 1 M, 2 VI 1973, Parsons (MCZ) [microvial contains cleared genitalia in glycerin]; Stowe, 1 M, 2 F, 18 VI 1927, Alexander (USNM) [M slide mounted genitalia, wing, remainder pointed].

DISTRIBUTION. This species has a wide geographic distribution (Figure 3B), but is rare throughout its range even when the likely habitat is surveyed carefully. Populations have been found in the plains of Canada in Manitoba and Saskatchewan, south into South Dakota and Michigan. Eastern populations have been collected from New York, Vermont and Maine. The ENM performed well (AUC_{Train} = 0.8466, AUC_{Test} = 0.7542, test omission = 0.0625), but may have overpredicted, or produced false positives given the extremely broad distribution in areas where N. caudifera has not been detected despite widespread sampling (e.g, southern Appalachians). The low sample size (N=16) may have overpredicted the potential distribution (Raxworthy et al. 2003, Anderson et al. 2005) or perhaps the historical distribution. This species seems to be locally abundant when discovered (Gathmann and Williams 2006), but regionally rare given the relative low abundance of
specimens in museum collections and the few populations encountered during surveys in 2009. Due to lack of historical biological data on this species, if its rarity is due to biotic exclusion (competition, predation, etc. not modeled through ENM), habitat alteration or historical biogeographic effects is unclear. The factors determining the current broad distribution remain elusive.

PHENOLOGY. Especially when compared to the other species in the genus, *N. caudifera* has a relatively short flight period lasting from May 20 in Aweme, Manitoba through July 12 near Harney Peak, South Dakota (Figure 4B). The majority of the collections were made in late May in Michigan. There is no effect of latitude (avg=44.9, SD=2.8) on Julian day (avg=155.4, SD=16.1) ($r^2 = 0.02$; P=0.0581).

BIOLOGY. Little to no biological information is known for this species. However, a recent analysis of coldwater springs sampled 10 adult individuals from a variety of habitats near Toronto, Ontario (Gathmann and Williams 2006). Their multivariate analysis indicated that the immature of *N. caudifera* are most likely found in cold-water, high-flow conditions. *Neophylidorea caudifera* adults are generally found near aquatic habitats (Young and Gelhaus 2000). Altitudes range from 195 m in Michigan to 500 m in New York.

*Neophylidorea flavapila* (Doane 1900)

*Limnophila flavapila* Doane, 1900: 190 [original designation].

*Limnophila strepens* Alexander, 1916: 532 [original designation]; 1965: 66 [synonymy with flavapila]

*Limnophila* (*Phylidorea*) aleutica Alexander, 1920a: 198 [original designation]. **NEW SYNONYM**

*Limnophila* (*Phylidorea*) tepida Alexander, 1926: 119 [original designation]. **NEW SYNONYM**

Alternative spelling: *flavipila*

DIAGNOSIS. Male median lobe of the ninth tergite bulbous. Ventral parameres hooked apically. Aedeagal filaments long and slender with the center filament strongly bent at the apex. The central confusion surrounding this species has likely been due to the great variability in coloration of the thorax and abdomen (Alexander 1943).
DESCRIPTION. **Adult. Male** (Figure 5). **Measurements** (N=54): body length: 9.9 mm (8-12.5 mm); wing length: 10.26 mm (8.5-12 mm). **Wing.** Occasional shading along cord, apically and at base of Rs; Rs spurred in some individuals; stigma prominence variable among individuals. **Abdomen.** Yellow to brown with varying combinations of dark and light colorations on terminal segments. **Hypopygium.** Dorsal gonostylus bent slightly at about 1/3 the length; ventral gonostylus finger-like extension at apex nearly straight; large knob on gonocoxite at base of dorsal gonostylus; patch of setae on inner surface of gonocoxite; aedeagus often heavily sclerotized, center filament strongly bent apically, filaments long and slender, lateral filaments often fluted apically; ventral paramere with small hook apically, otherwise slender and elongate, slightly broadened basally; median lobe of 9T large, bulbous, slightly divided medially; lateral processes on 9T present, but severely reduced, often visible laterally. **Female.** **Measurements** (N=11): body length: 10.45 mm (9-12.5 mm); wing length: 10.55 mm (9-12 mm). Larger than males, but morphologically similar unless noted otherwise. **Abdomen.** Yellow on all segments except occasionally a brown terminal ring on 8th segment. **Pupa.** Undescribed. **Larva.** See description of larva in Chapter 2.

**TYPE MATERIAL.** According to the original description of *flavapila*, one male and three females were collected from Pullman, Washington (Doane 1900) and would be today considered syntypes (Alexander 1967). No type specimens of *N. flavapila* were located and the original description is incomplete. However, the identity of *N. flavapila* is well established. Alexander sketched a detailed depiction of *N. flavapila* genitalia in The Crane Flies of California (Figure 374, 1967) which matches the description that follows. Therefore, according to the International Code of Zoological Nomenclature (ICZN 1999, art.75), a neotype designation is not necessary. The *E. tepida* holotype (male) is not listed below because the label simply states “Colorado” with no additional information (USNM).

**OTHER MATERIAL EXAMINED** (N=183 adults, 1 larva). **Canada. Alberta:** Banff, 1 F, 29 V 1922, Garrett (USNM); as in preceding but 1 M, 18 VI 1922 [slide mounted genitalia, wings, remainder pointed]; Kananaskis, Environmental Science Centre, 1 M, 18 VI 1968, Pritchard (USNM) [slide mounted genitalia, legs, wing, head, remainder pinned]; as in preceding, but 26 VI 1969;
British Columbia: Forbidden Plateau, 1 F, 8 VIII 1950, Guppy (UMMZ) [identified by DFA]; as in preceding, but 9 VIII 1950; Forbidden Plateau, 1 M, 10 VIII 1950, Guppy (UMMZ); as in preceding but 11 MM, 2 FF, 11 VIII 1950 [1 M with microvial containing cleared genitalia in glycerin; study specimen #5533 includes slide mounted genitalia, wing, remainder pointed; females identified by DFA]; as in preceding but 1 M, 12 VIII 1950; Hollyburn Ridge, 3000 ft., 1 M, 28 VI 1931, Leech (USNM); Mercer Lake, Graham Island, 2 MM, 21 VII 1988, Brodo (FB); Moose Meadows, Mt Revelstoke National Park, N51.03434° W118.16234°, 5200 ft., 6 MM, 12 VII 2008, J. Petersen (ISIC) [1 M pointed, remainder in 95% EtOH]; Port Machell, 1 M, 5 VII 1952, Fender (UMMZ) [microvial contains cleared genitalia in glycerin]; Vancouver, 1M, 28 VI 1931, Leech (USNM); as in preceding, but 4 V 1949, Guppy (UMMZ); Vancouver Island, 1 F, 10 V 1949 Guppy (UMMZ) [identified by DFA]; as in preceding, but 26 V 1949; as in preceding, but 2 FF, 2 IX 1949; as in preceding, but 1 F, 13 IX 1949; as in preceding but 17 X 1950.

UNITED STATES. Alaska: Admiralty Island, 2 MM, 1 F, 23 VI 1933, Shepard (UMMZ); as in preceding but 1 M, 26 VI 1933; as in preceding but 1 M, 1 F, 1 VII 1933; as in preceding but 1 F, 21 VII 1933; as in preceding but 1 M, 1 F, 22 VII 1933; as in preceding, but 4 FF, 25 VIII 1933; as in preceding but 2 FF, 26 VIII 1933; Juneau, 1 M, 7 VI 1988, Brodo (FB) [marsh, bog]; as in preceding, but 2 FF, 14 VI 1988; Katmai, 2 MM, 10 VI 1919, J.S. Hine (USNM) [aleutica paratypes, slide mounted genitalia, wing, antennae, leg, remained pointed]; Valdez, 1 M, 11 VII 1949, Miller (UMMZ) [slide mounted genitalia only]; California: Grass Lake, El Dorado National Forest, N38.7937° W119.9588°, 7700 ft., 8 MM, 2 FF, 18 VI 2007, J. Davis and M. Petersen (ISIC) [2 FF and 4 MM pointed, remainder in 95% EtOH]; Colorado: Beaver Creek, Rio Grande Co., 10 000 ft., 1 M, 21 VI 1972, Wirth (USNM) [Malaise trap]; Gothic, 9500 ft., 1 M, 4 VII 1947, Unknown (USNM); Lefthand Bog, N40.06° W105.56°, 10 700 ft., 2 MM, 11 VII 1979, Brodo (FB); Middle Fork Williams Fork River, 7 MM, 19 VII 1929, Clagg (USNM); Mt. Res. Stn., N40.03° W105.53°, 9500 ft., 1 M, 24 VI 1979, Brodo (FB); Rainbow Lakes, N40.016° W 105.583°, 10 100 ft., 2 MM, 10 VII 1979, Brodo (FB) [microvial contains cleared genitalia in glycerin]; Slate Creek Ranger Station, 8220 ft., 1 M, 10 VII 1929, Clagg (USNM); Idaho: Alturas Lake Creek, Sawtooth National Forest, N43.9824° W114.8452°,
6900 ft., 4 MM, 1 F, 5 VII 2008, J. Petersen (ISIC) [3 MM pointed, remainder in 95% EtOH]; Big Wood Wetland, Sawtooth National Forest, N43.82706° W114.62204°, 6870 ft., 2 MM, 2 FF, 4 VII 2008, J. Petersen (ISIC) [1 M pointed, remainder in 95% EtOH; 1 female identified by molecular analysis, 1 female identified by DFA]; Montpelier, 2 MM, 1 VII 1942, Alexander (USNM); Redfish Lake Creek, Boise National Forest, N44.16608° W114.90108°, 6900 ft., 1 M, 5 VII 2008, J. Petersen (ISIC) [95% EtOH]; Robinson Lake, 2 MM, 2 FF, 1 V 1959, B.A. Foote (USNM) [reared from pupae]; Stanley Lake Creek, Boise National Forest, N44.25339° W115.0074°, 6900 ft., 2 MM, 5 VII 2008, J. Petersen (ISIC) [95% EtOH]; Vader Creek, Boise National Forest, N44.3469° W115.1206°, 6900 ft., 1 M, 5 VII 2008, J. Petersen (ISIC) [95% EtOH; identified to species through molecular phylogenetics, Chapter 3]; Nevada: Wells, 1 M, 5 VI 1915, McVanDuzee (USNM) [microvial contains cleared genitalia in glycerin]; as in preceding, but 1 F, 6 VI 1915; Oregon: Buttermilk Creek, Malheur National Forest, N44.12542° W118.44782°, 5100 ft., 5 MM, 1 F, 6 VII 2008, J. Petersen (ISIC) [4 MM pointed, remainder in 95% EtOH]; Catlow Valley, 4553 ft., 1 M, 22 V 1950, Fender (USNM) [microvial contains cleared genitalia in glycerin]; Crane Flats, Wallowa Whitman National Forest, N44.8807° W118.4038°, 5500 ft., 2 MM, 28 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Crane Prairie, Deschutes National Forest, N43.8278° W121.7736°, 4500 ft., 2 MM, 1 L, 27 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Deschutes River Nr. Redmond, 2800 ft., 7 MM, 1 F, 22 V 1950, Fender (USNM) [pointed; 1 M with microvial containing cleared genitalia in glycerin]; Fish Lake, Steens, 7200 ft., 2 MM, 1 F, 13 VII 1953, Baker (USNM); Little Cultus Lake, Deschutes National Forest, N43.8004° W121.8781°, 4800 ft., 1 M, 27 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Mud Lake, N44.9647° W118.2331°, 7100 ft., 7 MM, 2 FF, 28 VI 2007, J. Davis and M. Petersen (ISIC) [3 MM and 1 F pointed, remainder in 95% EtOH]; Olive Lake, Umatilla National Forest, N44.7830° W118.5955°, 6100 ft., 10 MM, 27 VI 2007, J. Davis and M. Petersen (ISIC) [4 MM pointed, one with microvial containing cleared genitalia in glycerin, remainder of specimens in 95% EtOH]; Page Springs Campground, N44.9647° W118.2331°, 3600 ft., 1 M, 2 VI 2008, G.W. Courtney (ISIC) [95% EtOH]; Trout Meadows, 5500 ft., 1 M, 9 VII 1967, Unknown (USNM) [microvial contains cleared genitalia in glycerin]; Utah: Charleston, 2 MM, 14 VIII 1943, Knowlton and Maddock (USNM); Garden
City, 2 MM, 25 VIII 1938, Knowlton and Hardy (USNM) [one specimen with slide mounted genitalia, wing and leg, remainder pointed]; as in preceding but 1 M, 11 VIII 1942, Knowlton [slide mounted genitalia and wing, remainder pointed]; Mantua, 1 M, 4 VIII 1938, Knowlton and Stains (USNM); Strawberry Reservoir, along stream, 7600 ft., 2 MM, 25 VII 1945, Knowlton (USNM); Washington: Seattle, 1 M, 1 F, date unknown, Piper (USNM); Wyoming: Cascade Canyon, 8500 ft., 1 M, 9 VII 1941, Alexander (USNM) [2 additional specimens lack genitalia]; Conservation Research Fen, Jackson Co., N43.47807° W110.81519°, 7800 ft., 2 MM, 3 VII 2008, J. Petersen (ISIC) [95% EtOH]; Gibbon River, 6000 ft., 2 MM, 17 VI 1952, Rogers (USNM) [study specimen #6225 entire specimen slide mounted]; Madison Jct., Yellowstone Pk, 0.7 m.e. on Gibbon R., 6800 ft., 2 MM, 17 VI 1952, Hayden (USNM); Moran Bog, 2.4 mi N of Moran, 1 M, 5 VII 1941, Alexander (USNM); as in preceding but 2 FF, 2 VII 1941; Nash Fork, 2 MM, 9 VII 1981, Teale (USNM); Pinnacle Heights Summer Camp, Shoshone National Forest, N43.7179° W109.9575°, 8150 ft., 2 MM, 1 VII 2008, J. Petersen (ISIC) [95% EtOH]; Snowy Pass, HWY 130, UTM 13T 0382635 4575288, 3 MM, 2 FF, 18 VII 2009, B. Danielson (ISIC) [95% EtOH]; Sylvan Lake, Yellowstone National Park, 8000 ft., 3 MM, 21 VI 1941, Alexander (USNM) [1 M with slide mounted genitalia, wing and leg, remainder pointed; 1 M with microvial containing cleared genitalia in glycerin].

DISTRIBUTION. This species is widely distributed in the western United States and north along the Pacific coast into British Columbia and coastal Alaska (Figure 6B). Over much of its range, it is sympatric with N. columbia in terms of known localities (Figure 6A). Populations are confined to high-altitude, montane regions including the Sierra Nevada’s (California and Nevada), Cascades (Oregon and Washington), Blue Mountains (Oregon), Rocky Mountains (Wyoming, Colorado, Alberta, and British Columbia), Ruby Mountains (Nevada), Wasatch Range (Utah), and Sawtooth Mountains (Idaho).

Ecological niche modeling developed a robust potential distribution of N. flavapila (AUC_{Train} = 0.8484, AUC_{Test} = 0.7663, test omission = 0.0992). Visually, the minimum training presence threshold binomial distribution map shows a good fit to the known localities (N=51). From sampling in 2007-2009, populations are difficult to find within regions such as the Sierra Nevada’s. But populations in
other regions (e.g., Sawtooth Mountains) were located in 2008 based on ENM created using preliminary data prior to collecting.

PHENOLOGY. The phenology of *N. flavapila* is the broadest of all species and ranges from April 28, in Cuyamaca State Park, California to October 4 in Vancouver, British Columbia (Figure 4D). There is a significant positive effect of latitude (avg=46.7, SD=6.0) on Julian day (avg=188.5, SD=29.8) ($r^2 = 0.03; P<0.0344$).

BIOLOGY. Alexander describes this species from bogs and boggy areas in Engelmann Spruce forests (1945a), and lodgepole forest (WY). Herbaceous layer includes plants such as white bog orchids, elephant’s head (figwort), green false hellebore. *Sphagnum* bogs are also a preferred habitat type for this species. Water temperatures of the nearby surfaces ranged between 8.0-14.5°C and a quite variable pH of 5.8-8.0. This species has been collected using Malaise traps, but is most frequently collected by sweeping emergent vegetation along slow moving rivers, braided wetlands and fens. Altitudes vary from 900 m in Oregon and Washington to over 3000 m in Colorado, although *N. flavapila* was collected by Alexander at Peavine Ridge in Oregon and indicated an altitude of 60 m at the collecting location.

This is the only species to have a direct association between the larva and adult. A single larva was collected at near Crane Prairie along Snow Creek in Deschutes National Forest, Oregon on June 27, 2007 at 1400 m elevation. The creek was relatively fast flowing, but there was a great deal of riparian area along the edges with stands of emergent vegetation. The larva was collected by sifting clumps of emergent vegetation collected in <1 m of standing water along the creek edge through a medium mesh. Based on this record, it appears that this particular species is fully aquatic as larva, inhabiting shallow lakes and small creek edges. The larva contained nearly whole Chironomidae and Ceratopogonidae larvae in its digestive tract and is therefore considered predaceous. Adults of this species were collected simultaneously (2 males) and all three individuals were sequenced for two mitochondrial genes (COI and COII: Chapter 3). Haplotype network analysis using parsimony identified the larva as having an identical genetic sequence to that of one adult.
(T5407b and T5407c) for both genes (Chapter 3). Therefore, we were able to associate the larva with an adult of the same species.

SYNONOMY. Although never explicitly stated, the implicit difference between *N. flavapila* and *N. tepida* as determined by Alexander seems to be one of geography. Specimens identified by Alexander as *N. flavapila* are from California, Oregon, Idaho, Washington, and British Columbia whereas *N. tepida* specimens are from Colorado (holotype), Wyoming, Utah, and Alberta. Alexander describes *N. tepida* as “closely allied to *L. (P.) flavipila* Doane, 1900” (1945a).

*Neophylidorea vanronea, sp. nov.*

DIAGNOSIS. *Neophylidorea vanronea* has unique genitalia, and is clearly distinguished from other species in the genus based on characters of the ventral parameres: heavily sclerotized with a flat, toothed aspect distal to the large, strongly hooked terminus.

DESCRIPTION. **Adult.** **MALE.** Genitalia (Figure 7A): Median lobe on 9th tergite with width equal to length (as in *N. adusta*) pubescent, dorsal process more reduced. Inner gonostylus angled strongly, slightly anterior to mid-length, base wide. Ejaculatory apodeme appears smaller than in other species. Medial aedeagal sheath bent apically at 90 degrees (similar to *N. tepida*), moderately sclerotized. Ventral parameres broad laterally, terminating in large, sickleshaped process, with teeth along margin of plate just prior to sickle, heavily sclerotized. **FEMALE.** Undescribed. **Larva.** Undescribed. **Pupa.** Undescribed.

TYPE MATERIAL. **Holotype.** Male, collected by J. Speed Rogers on VI-2-1948 in Iosco County, Michigan (UMMZ). Slide mounted genitalia and wing in Canada balsam. Slide indicates study specimen #4146, but the remainder of the specimen was not located. Also noted on the slide is “#23” which may indicate a study site, but this information was not recovered. Originally identified as *Limnophila (P.) neadusta* Alex. by Rogers. **Paratype.** Same data as the holotype, except collected on VI-3-1948. Slide specimen includes only male genitalia and indicated as study specimen #3917 and as above regarding a potential study site, but “#25”. Originally identified as *Limnophila (P.) caudifera* Alex. by Rogers.
DISTRIBUTION. This species has been collected only from Iosco County, Michigan. Joseph Speed Rogers took meticulous collection notes that are stored at UMMZ. Using collection information from the label, we were able to identify more specific localities. Both the holotype and paratype were collected at Gordon Creek, “2.5 mi south of Lumberman’s Monument” in Huron National Forest. The senior author collected Neophylidorea from Michigan, including in Iosco County in 2009, but was unable to locate additional specimens of N. vanronea sp. nov.

BIOLOGY. Rogers described the habitat as “alder, spruce, tamarack grown low valley of trout brook with numerous mossy seepage areas”. He also noted the co-occurring crane flies Tipula (Trichotipula) oropezoides Johnson, Dicranota Zetterstedt and Eloeophila.

ETYMOLOGY. The species-group name is in remembrance of Ron VanNimwegen, a dear friend of the senior author and a great naturalist and scientist.

REMARKS. Although existing specimens do not allow comparisons between species regarding any feature other than male genitalia, we are confident that additional specimens will support our decision to recognize existing material as a new species. Many features of the head, thorax and abdomen of Neophylidorea species are morphologically similar across species.

Why Rogers would have identified the two specimens as he did (one as N. caudifera the other as N. neadusta) is unclear. At the time the specimens were collected, there was a key to species of Connecticut (Alexander 1942) that included sympatric Neophylidorea species, which he likely used to identify these specimens. Given the morphological uniqueness of the male genitalia of this species as compared to all species of Neophylidorea but why Rogers did not pursue description of a new species is unclear.

Neophylidorea neadusta (Alexander 1927)

Limnophila (Phylidorea) neadusta Alexander, 1927a: 110 [original designation].

DIAGNOSIS. Similar to N. adusta, but differing in the morphology of the male genitalia: ventral parameres bearing a lateral spine and aedeagal apex strongly recurved.
DESCRIPTION. Adult. MALE (Figure 7B). Measurements (N=1): body length: 8.5 mm; wing length: 9 mm. Wing. Apical and cord clouding, M, approximately equal to its petiole. Legs. Femora yellow, tips darkened. Hypopygium. Dorsal gonostylus stout, sharply angled; knob on gonocoxite at base of dorsal gonostylus absent; lacks patch of setae on inner fleshy surface of gonocoxite; median lobe of 9T slightly divided medially, lateral processes less pronounced than in other species (*N. adusta, N. columbiana*); ventral paramere with lateral spine, aedeagus long and slender, apex strongly curved/bent. FEMALE. Undescribed. Pupa. Undescribed. Larva. Undescribed.

TYPE MATERIAL. Holotype. New York, Keene Valley, 1 M, 14 VII 1920, Notman (USNM) [slide mounted genitalia and wing, carcass pinned].

OTHER MATERIAL EXAMINED. None.

DISTRIBUTION. Known only from a single male collected in Keene Valley, New York.

REMARKS. This species is unique morphologically. Although morphologically most similar to the sympatric species *N. adusta*, the spine along the ventral paramere and a bent apex to the median aedeagal filament make it a distinct species. Considerable collecting has been done in the region surrounding the holotype locality, although this area of the Adirondacks can be quite remote and not easily accessed. Additional collecting in Essex County, New York may reveal additional populations.

*Neophylidorea columbiana* (Alexander 1927)

*Limnophila (Phylidorea) columbiana* Alexander, 1927b: 12 [original designation]

*Limnophila (Phylidorea) snoqualmiensis* Alexander, 1945b: 94 [original designation]. NEW SYNONYM

**SYNONYM**

*Limnophila (Phylidorea) pacalis* Alexander, 1949a: 156 [original designation]. NEW SYNONYM

*Limnophila (Phylidorea) nevadensis* Alexander, 1958: 218 [original designation]. NEW SYNONYM

*Limnophila (Phylidorea) brevifilosa* Alexander, 1959: 50 [original designation]. NEW SYNONYM

*Limnophila (Phylidorea) burdicki* Alexander, 1964: 120 [original designation]. NEW SYNONYM

*Limnophila (Phylidorea) olympica* Alexander, 1949b: 316 [original designation]. NEW SYNONYM
DIAGNOSIS. Distributed throughout western North America. Morphologically very similar to *N. adusta*; central aedeagal filament straight, but slightly expanded apically; flattened dorsal lobe, invaginated medially with dense setae; dorsal gonostylus strongly bent, broad basally. Ventral paramere shape varies continuously from tapering to an acute tip to individuals with gradually narrowing and subacute tip.

DESCRIPTION. **Adult.** MALE (Figure 8). **Measurements** (N=31): body length: 9.79 mm (7-12.5 mm); wing length: 9.97 mm (9-12 mm). **Legs.** Femur with varying proportions of yellow and brown/black. Abdomen. Yellow with varying combinations of yellow and brown rings on the terminal 3 segments. **Hypopygium.** Abundant, elongate, yellow to brown setae on gonocoxite, ventral and dorsal portions of 9T; invagination of the sclerotized outer surface of gonocoxite into fleshy inner surface (Figure 8A); aedeagal complex usually heavily melanized; central aedeagal filament fluted apically; median lobe folded within hypopygium on some dried specimens, median lobe flush with the ninth tergite surface on other specimens, short stout setae dense and pointed apically, usually deeply invaginated medially. FEMALE. **Measurements** (N=23): body length: 10.38 mm (6.5-14 mm); wing length: 10.17 mm (7.5-13 mm). **Pupa.** Undescribed. **Larva.** Undescribed.

**TYPE MATERIAL.** Holotype. British Columbia, Prince Rupert, 1 M, 17 VI 1919, Dyar (USNM).

**OTHER MATERIAL EXAMINED (N=170).** CANADA. British Columbia: Lake Diane Provincial Park, 1 M, 27 VII 1988, Brodo (FB) [microvial contains cleared genitalia in glycerin]; Forbidden Plateau, 1 F, 8 VIII 1950, Guppy (UMMZ) [identified by DFA]; as in preceding, but 10 VIII 1950; as in preceding, but 1 M, 1 F, 11 VIII 1950 [male is study specimen #5535; female identified by DFA]; Vancouver Island, Port Hardy, 1 M, 13 VI 1952, Fender (UMMZ) (microvial contains genitalia cleared in glycerin); Vancouver, 1 M, 8 VII 1950, Guppy (UMMZ) [pointed]; Vancouver Island, 1 F, 22 VI 1949, Guppy (UMMZ) [identified by DFA]; as in preceding, but 13 VII 1949; Vancouver Island, Mackenzie Lake, N49.7° W125.33°, 3855 ft, 1 M, 25 VII 1994, Goulet (FB) [moist meadow, pan trap]; Wellington, 1 M, 7 VI 1957, Guppy (USNM); Yakoun Lake, 1 M, 20 VII 1988, Brodo (FB) [microvial contains cleared genitalia in glycerin].
UNITED STATES. Alaska: Juneau, 1 M, 26 VI 1988, Brodo (FB) [microvial contains cleared genitalia in glycerin]; California: Bartle, 4 MM, 17 VI 1959, Byers (KSU) [one male specimen lacks genitalia, another pointed with microvial containing cleared genitalia in glycerin]; Big Pine Co., 4 FF, 11 VII 1957, Alexander (USNM) [brevifilosa paratypes; identified through DFA]; Cottonwood Creek Campground, 39.5434°N 120.3185°W, 1800 ft., 3 MM, 4 FF, 9 VII 2009, J. Petersen (ISIC) [95% EtOH]; Gumboot Lake, Shasta Trinity National Forest, 41.2105°N 122.5079°W, 6000 ft., 2 MM, 24 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Intake Camp, Bishop Creek, 1 M, 8 VII 1957, Alexander (USNM) [brevifilosa holotype]; Kangaroo Lake, Klamath National Forest, N41.3299° W122.6401°, 6300 ft., 1 M, 24 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Lassen National Park, 3 MM, 15 VI 1959, Byers (KSU) [1 M specimen with microvial containing cleared genitalia in glycerin]; Mt. Shasta, 1 M, 2 FF, 18 VI 1959, Byers (KSU); Plantation, 4 mi west of town, 1 M, 1 V 1958, Burdick (USNM) [burdicki paratype]; as in preceding but 1 M, 8 V 1958 [slide mounted genitalia, remainder pointed]; Sierraville, 4.8 mi SE of town, 2 MM, 3 FF, 14 VI 1959, Byers (KSU) [1 M specimen with microvial containing cleared genitalia in glycerin]; Susanville, Lassen Co., 1 F, 24 VII 1911, Unknown (ANSP) [identified by DFA]; as in preceding but 2 FF, 22 VII 1911; Truckee, 6000 ft., 1 M, 1 F, 4 VII 1953, Alexander (USNM) [pointed, M lacking genitalia]; Colorado: Durango, 1 M, 19 V 1913, Oslar (ANSP); Idaho: Big Wood Wetland, Sawtooth National Forest, N43.82706° W114.62204°, 6870 ft., 1 M, 4 VII 2008, J. Petersen (ISIC) [95% EtOH]; Redfish Lake Creek, Boise National Forest, N44.16608° W114.90108°, 6900 ft., 2 MM, 1 F, 5 VII 2008, J. Petersen (ISIC) [95% EtOH]; Nevada: Lake Tahoe, east side of lake, 6800 ft., 3 MM, 3 VII 1953, Alexander (USNM) [nevadensis holotype]; Ormsby Co., 2 MM, 4 FF, 20 VII 1950, Alexander (USNM); Spooners Summit, 2 MM, 3 VII 1953, Alexander (USNM) [nevadensis paratypes, pointed together on one pin, with microvial containing cleared genitalia from one specimen in glycerin]; Oregon: Blue Creek, Siskiyou National Forest, 1 F, 9 VIII 1948, Fender (USNM); Castle Rock, 1 M, 13 V 1949, Fender (USNM); Crane Flats, Wallowa Whitman National Forest, N44.8807° W118.4038°, 5500 ft., 1 M, 1 F, 28 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Deer Creek, Mt. Hood, 3 MM, 15 VII 1954, Fender (MCZ) [microvial contains cleared genitalia in glycerin]; Elk Lake, Century Drive, Cascade
Mts., 4900 ft., 1 M, 3 VII 1948, Fender (USNM); as in preceding but 1 M, 1 F, 5 VIII 1948 [male specimen with slide mounted genitalia, head, legs, wing, remainder pointed]; as in preceding but 1 M, 6 VIII 1948 [slide mounted genitalia, head, legs, wing, remainder pointed]; Hood River Meadows, Mt. Hood, 4480 ft., 1 M, 3 VII 1948, Fender (USNM); as in preceding but 11 MM, 3 FF, 17 VII 1947, Alexander (USNM); as in preceding but 5 MM, 1 F, 31 VII 1948, Fender (USNM); as in preceding but 3 MM, 1 F, 8 VIII 1946, Fender [specimens pointed, 1 M lacks genitalia, 1 M with microvial containing cleared genitalia in glycerin]; Horsethief Meadows, 2 MM, 18 VII 1947, Fender (USNM) [specimens pointed, one with microvial containing cleared genitalia in glycerin]; Langdon Lake, Blue Mts., 4990 ft., 1 F, 17 VIII 1948, Alexander (USNM) [paca]llotype; as in preceding, but 1 M [paca]lotype]; as in preceding but 17 VII 1948, Lane [paca]lotype, slide mounted genitalia]; Little Cultus Lake, Deschutes National Forest, N43.8004° W121.8781°, 4800 ft., 2 MM, 1 F, 27 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH, 1 female identified by molecular analysis]; McMinnville, Yamhill Co., 1 M, 26 V 1948, Fender (USNM); as in preceding but 1 M, 29 V 1948; Olive Lake, Umatilla National Forest, N44.7830° W118.5955°, 6100 ft., 1 M, 1 F, 27 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Peavine Ridge, Station 1, Willamette Valley, 210 ft., 3 MM, 14 V 1945, Fender (USNM); as in preceding but 1 M, 15 V 1945; as in preceding but 1 M, 18 V 1945; as in preceding but 1 M, 19 V 1945; as in preceding but 2 MM, 22 V 1947 [specimens pointed, one with microvial containing cleared genitalia in glycerin]; as in preceding but 2 MM, 31 V 1945 [both specimens lacking genitalia, one with microvial contains cleared genitalia in glycerin]; as in preceding but 3 MM, 1 F, 2 VI 1945; as in preceding but 1 M, 8 VI 1946; as in preceding but 1 M, 10 VI 1946; Salmon River, Mt. Hood Village, 1 M, 30 VII 1948, Fender (USNM) [microvial contains cleared genitalia in glycerin]; Sphagnum Bog, N42.9995° W122.2524°, 5400 ft., 3 MM, 1 F, 26 VI 2007, J. Davis and M. Petersen (ISIC) [1 M pointed, remained in 95% EtOH]; Spring Creek, Witman National Forest, Blue Mts., 3900 ft., 1 M, 24 VI 1948, Alexander (USNM) [slide mounted genitalia, wing, legs and antennae, remainder pointed]; as in preceding but 1 M, 1 F, 25 VII 1945, Baker [M specimen lacks genitalia, no slide recovered]; Timberline Lodge, Mt. Hood, 1 M, 15 VII 1954, Fender (UMMZ) [microvial contains cleared genitalia in glycerin]; Tollgate, Langdon Lake Post Office, Blue Mts., 4990 ft., 4 FF, 17 VIII 1948, Alexander
(USNM) [pacalis paratypes]; Trout Creek Meadows, Baker Co., 2 MM, 24 VI 1956, Baker (USNM); Trout Meadows, Grant Co., 5500 ft., 1 M, 9 VII 1967, Unknown (USNM) [microvial contains cleared genitalia in glycerin]; Unnamed Meadow, Wallowa Whitman National Forest, N44.9642° W118.2554°, 7100 ft., 2 MM, 28 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; **Washington**: Black Creek Forest Camp, Gifford Pinchot National Forest, 2800 ft., N45.89598° W121.85951°, 4 MM, 19 VII 2008, J. Petersen (ISIC) [2 pointed, 2 in 95% EtOH]; Ohanapeosh Springs, Rainier National Park, 1800 ft., 2 MM, 28 VII 1953, Alexander (USNM); as in preceding but 1 M, Fender (UMMZ) [entire specimen slide mounted in Canada balsam]; Snoqualmie Pass, 3000 ft., 2 MM, 29 VI 1924, Melander (USNM) [snoqualmiensis holotype and paratype with slide mounted genitalia in Canada balsam]; as in preceding but 1 F, 22 VII 1950, Byers (KSU); **Wyoming**: Beaver Dick Lake [String Lake], Teton National Park, 6820 ft., 7 MM, 4 FF, 7 VII 1941, Alexander (USNM) [2 MM with associated slide mounted genitalia wing and leg, remainder pointed]; Medicine Bow National Forest, unnamed beaver creek, 8450 ft., N41.3427° W106.4912°, 2 MM, 2 FF, 13 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Twin Lakes, 1 M, 1 F, 10 VII 1923, Melander (USNM) [microvial contains cleared genitalia in glycerin]; Yellowstone National Park, 2 MM, 1 F, 11 VII 1942, Alexander (USNM).

**DISTRIBUTION.** This species is widely distributed in the western United States and north along the Pacific coast into British Columbia (Figure 6A). Populations are confined to high altitude montane regions including the Sierra Nevadas (California), Cascades (California), Blue Mountains (Oregon) Rocky Mountains (Wyoming), and Sawtooth Mountains (Idaho). This species is often collected sympatrically with *N. flavapila* (Figure 6B). Sympatric sites for *N. flavapila* and *N. columbiana* include: Forbidden Plateau, British Columbia; Olive Lake, Oregon; Redfish Lake Creek, Idaho.

Ecological niche models were built based on 38 localities and produced a relatively good approximation of the potential distribution ($AUC_{\text{Train}} = 0.8976$, $AUC_{\text{Test}} = 0.8133$, test omission = 0.1440). However, this species had the highest test omission and again there may be some overprediction, especially to the north (Figure 6A). The northern Rocky Mountains were sampled in 2008, and *N. flavapila* populations were collected, but no *N. columbiana* populations were recovered.
PHENOLOGY. As with other species, the period of adult emergence has a broad distribution (Figure 4C). Collections of adults have been made as early as May 1, in Plantation, California and as late as August 17 in Langdon Lake, Oregon. There is a significant positive effect of latitude (avg=44.7, SD=3.4) on Julian day (avg=187.0, SD=30.7) (r² = 0.10; P<0.0001).

BIOLOGY. This species was collected from alder and willow habitats with meandering streams. Regardless of the dominant tree cover, the nearby water sources always included a diversity of emergent aquatic vegetation especially grasses and sedges. Open habitats contained various wetland-type, herbaceous plants such as shooting star, bunchberry dogwood, and green false hellebore. *Neophylidorea columbiana* was occasionally collected in more serpentine fens such as near Kangaroo Lake in Klamath National Forest, Oregon where pitcher plant was the abundant vegetation along with rushes and sedges. Water temperatures of the nearby surfaces ranged between 10.1-14.0°C and a pH range of 5.5-8.3. *Neophylidorea columbiana* has been collected using pan traps, but more generally is collected by sweeping emergent vegetation along pond edges and wetlands.

SYNONYMY. This work synonymyzes six previously described species into a single species with a vast range of continuous morphological variability. In particular, the ventral parameres are quite variable as indicated in the diagnosis and description (Figure 9E and F). After studying and comparing many specimens, a continuous morphological cline emerged between the two extremes (acute – Figure 9E and subacute – Figure 9F). The holotypes of the previously described species represent the morphological variability of the ventral parameres. *Neophylidorea brevifilosa* is on the extreme end of the spectrum with an acute ventral paramere. Few specimens approximate this acute condition. Two populations with acute ventral parameres were collected and analyzed as part of the molecular analyses used to delimit species of *Neophylidorea* (B1408a and B4407a; Chapter 3). These populations did not form a clade in the analyses performed. Likewise, morphological analyses using hierarchical clustering do not form discrete groups of specimens with more acute ventral parameres. As with the synonymized species in *N. flavapila*, the formerly described species now represented by *N. columbiana* appears to be somewhat representative of geographical separation.
However, the methods used elsewhere (Chapter 3) to delimit species of *Neophylidorea* do not indicate geographical structure associated with the morphological or molecular variability.

**REMARKS.** This species is very closely related to *N. adusta*. The differences are primarily based on geographic distribution. Although some morphology differs between species (e.g., wing shading) these differences are not necessarily fixed. For example, some specimens of *N. columbiana* from Wyoming have morphological features of *N. adusta* (wing clouding). Given the geographic separation and ecological niche differentiation (Chapter 3), these species are here considered distinct.

*Neophylidorea adusta* (Osten Sacken 1859)

*Limnophila adusta* Osten Sacken, 1859: 235 [original designation]

*Limnophila (Phylidorea) paeneadusta* Alexander, 1961: 85 [original designation]. **NEW SYNONYM**

*Limnophila terraenovae* Alexander, 1916b: 123 [original designation]. **NEW SYNONYM**

**DIAGNOSIS.** Male genitalia as in *N. columbiana*. Wings typically heavily shaded along the apical margin, cord and Rs. Distributed east of the Mississippi River. Most well known and frequently collected species of the entire group.

**DESCRIPTION.** **Adult.** **MALE (Figure 8, Figure 2A-E from Chapter 2).** **Measurements** (N=16): body length: 9.57 mm (8-11.5 mm); wing length: 9.56 mm (9-11 mm). **Antenna.** Flagellum exclusively yellow. **Thorax.** Yellow to brown. **Wings.** Spur on Rs present or absent; heavily shaded apically, along cord, base of *Rs*, fork of *Rs*, surrounding *dm*. **Legs.** Femora coloration ranging from nearly entirely brown to a terminal brown ring, remainder yellow. **Abdomen.** Segments 7, 8 and 9 coloration variable, each segment brown or yellow; median brown stripe variable. **Hypopygium.** Short setae on median lobe of 9T, degree of invagination of median lobe variable from shallow to deep, median membrane sometimes apparent on dried specimens; lateral process of 9T present; ventral gonostylus finger-like apex strongly hooked; long, yellow setae on gonocoxite; knob on gonocoxite at base of ventral gonostylus absent; large contiguous invagination of sclerotized portion of gonocoxite into inner surface of gonocoxite (Figure 8A); dorsal gonostylus wide at base, usually a knob at bend
ventral gonostylus apex strongly hooked; ventral parameres often situated with tips touching, but unconnected, apex subacute (Figure 9F); aedeagus usually light colored, occasionally darkened. FEMALE. *Measurements* (N=28): body length: 10.72 mm (9-13 mm); wing length: 10.63 mm (9-11 mm). Considerably larger than males, but morphologically similar unless noted otherwise. Abdomen. Usually brown/grey lateral darkening, segments 7 and 8 usually brown, otherwise yellow.

**Measurements** (N=28): body length: 10.72 mm (9-13 mm); wing length: 10.63 mm (9-11 mm). Considerably larger than males, but morphologically similar unless noted otherwise.

**Abdomen.** Usually brown/grey lateral darkening, segments 7 and 8 usually brown, otherwise yellow.

**Type Material.** Syntypes, two females and two males, pinned (MCZ). One female labeled “O. Sacken, L. adusta nob., #10191 “ the other labeled “#10191-2”. I propose here that the specimen with the most labeling as described above is deemed the lectotype. The second female specimen, “#10191-2” is hereby designated a paralectotype. Both specimens lack legs, but are otherwise intact. Additionally, two male specimens labeled simply “O. Sacken” were included as syntypes by Osten Sacken, but are here removed and identified as species near *Euphylydorea similis*. In the third full paragraph of the original description, Osten Sacken seems to be describing the two male specimens.

**Other Material Examined** (N=243). CANADA: *Newfoundland*: Aspen Brook, 300 ft., 2 MM, 17 VII 1961, Carson (USNM) [1 specimen slide mounted genitalia, wing and leg, remainder pointed]; New Brunswick: Caribou Plains, N45° 37.647' W65° 03.496', 1150 ft., 3 MM, 26 VI 2009, J. and M. Petersen (ISIC) [95% EtOH]; Chatham, 1 M, 23 VI 1929, Alexander (USNM); Nova Scotia: Amherst, 1 M, 14 VI 1962, Alexander, (USNM); Amherst Point Sanctuary, N45° 47.851’ W64° 15.072’, 100 ft., 2 MM, 2 FF, 22 VI 2009, J. Petersen (ISIC) [95% EtOH]; Blomidon Provincial Park – Jodrey Trail, N45° 15.885’ W64° 20.328’, 660 ft., 3 FF, 25 VI 2009, J. and M. Petersen (ISIC) [95% EtOH]; Digby, 1 M, 18 VI 1908, Russell (UMMZ) [study specimen #6622, slide mounted genitalia and wing, remainder pointed]; Dollar Lake Provincial Park, N44° 55.621’ W63° 19.079’, 350 ft, 2 MM, 2 FF, 22 VI 2009, J. Petersen (ISIC) [95% EtOH]; Long Lake Provincial Park, N44° 37.803’ W63° 39.499’, 150 ft., 2 MM, 2 FF, 24 VI 2009, J. Petersen (ISIC) [95% EtOH]; Ontario: Algonquin Park, 1 M, 27 VI 1941, Davies (UMMZ) [study specimen #2815, slide mounted genitalia and wings, remainder pointed]; Bells Corner, 1 F, 28 V 1972, Brodo (FB); Bells Corner, 1 F, 3 VI 1972, Brodo (FB); Larose Forest, N45° 22.213’ W75° 13.621’, 300 ft., 2 MM, 6 FF, 2 L, 7 VI 2009, Brodo and J. Petersen (ISIC)
[95% EtOH; L reared from eggs]; Mer Bleue – Ridge Rd, 1 F, 11 VII 1971, Brodo (FB); Mer Bleue, 8 MM, 3 FF, 20 V 1941, Rogers (UMMZ) [2 male study specimens #2756 and #2758, slide mounted genitalia and wing, remainder pointed]; Mer Bleue, 1 M, 28 V 1927, Walley (USNM); Ottawa, 1 M, 3 VI 1973, Brodo (FB) [microvial contains cleared genitalia in glycerin]; Ottawa, 1 M, 2 VI 1927, Curran (USNM); Sand Lake, 1 M, 28 VI 1926, F.P. Ide (USNM); Quebec: Alymer, N45.4° W75.851°, 3280 ft, 1 M, 1 F, 7 VI 1995, Brodo (FB); as in preceding but 2 MM, 1 F, 9 VI 1995; as in preceding but 1 M, 19 VI 1995; Black Lake, N45.4833° W75.8833°, 1 F, 23 VI 1968, Brodo (FB); as in preceding but 1 M, 15 VI 1967; La Verendrye Park, 47°02′0″N 76°32′0″W, 1 M, 6 VII 1976, Brodo (FB); Ramsay Lake, Gatineau Park, 1 M, 14 VI 1970, Brodo (FB); Ste. Foy, 1 F, 5 VI 1975, Brodo (FB); Tabletop, 1 M, 21 VI 1940, Brower (CNC) [microvial contains cleared abdomen in glycerin].

UNITED STATES: Connecticut: East River, 1 F, VIII 1911, Unknown (ANSP); Maine: Acadia National Park – Hio Rd., N44° 15.405′ W68° 20.043′, 100 ft., 3 MM, 19 VI 2009, J. Petersen (ISIC) [95% EtOH]; Augusta, 1 M, 8 VI 1944, Brower (USNM) [slide mounted genitalia, wing, leg, remainder pointed]; Caribou Bog, N44° 53.475′ W68° 44.295′, 165 ft, 1 M, 17 VI 2009, J. Petersen (ISIC) [95% EtOH]; Chester, 1 M, 29 V 1936, Brower (USNM) [paeneadusta holotype, slide mounted genitalia, wing, legs and antennae, remainder pointed]; Greenville, 792 ft., 1 M, 7 VII 1913, Conley (USNM); Mercer Bog, N44° 40.383′ W69° 56.196′, 320 ft, 1 M, 1 F, 17 VI 2009, J. Petersen (ISIC) [95% EtOH]; Mount Katahdin, 1 M, 28 VII 1951, Carson (USNM) [male slide mounted genitalia, wing, legs and head, remainder pointed]; Orono, 1 M, 31 V 1914, Parshley (USNM); Saco Heath Bog, N43° 32.48′ W70° 28.88′, 100 ft, 3 MM, 4 FF, 17 VI 2009, J. Petersen (ISIC) [95% EtOH]; Sunkhaze Meadow National Wildlife Refuge – Johnson Brook Trail, N44° 58.91′ W68° 31.06′, 250 ft, 1 M, 1 F, 18 VI 2009, J. Petersen (ISIC) [95% EtOH]; Massachusetts: Boston, 4 MM, 2 FF, VI, Melander (USNM); Brookline, 1 M, 06 IX 1908, Unknown (MCZ) [slide mounted]; Lake May, 1 M, 15 VI 1925, Alexander (USNM) [slide mounted genitalia, remainder pointed]; Michigan: Au Sable State Forest, N43° 37.427′ W84° 30.500′, 570 ft, 2 MM, 24 V 2009, J. Petersen (ISIC) [95% EtOH]; ES George Reserve, 1 F, 22 V 1938, Cantrall (UMMZ); as in preceding but 2 FF, 9 VI 1938, Rogers; as in preceding but 7 MM, 6 FF, 21 VII 1938; as in preceding but 2 MM, 4 VII 1938; as in preceding but 3 MM, 5 FF, 15 VIII 1937;
as in preceding but 4 MM, 6 FF, 10 VI 1938 [male study specimen #2140, slide mounted genitalia and wing, remainder pointed]; as in preceding but 6 MM, 6 FF, 14 VIII 1937 [male study specimen #2143, slide mounted genitalia and wing, remainder pointed]; as in preceding but 1 M, 12 VIII, 1937, THH (UMMZ); Emmet Co., 1 M, 1 F, 27 V 1960, Dreisbach (UMMZ) [male slide mounted genitalia, wing, legs and antennae, remainder pointed]; Gogebic Co. 1 M, 28 VII 1920, Rogers (UMMZ); Hanel Preserve, N45° 15.928' W84° 36.945', 780 ft., 2 MM, 29 V 2009, J. Petersen (ISIC) [95% EtOH]; Hillsdale Co., 1 M, 21 V 1969, Dreisbach (UMMZ); Huron Mountains, 1 M, 13 VII 1950, Rogers (UMMZ); as in preceding but 1 M, 1 F, 15 VII 1950; Iosco Co. 1 M, 1 F, 3 VI 1948, Rogers (UMMZ); Livingston Co., 1 M, VIII 8 1936, Rogers (UMMZ); Missaukee Co. 1 M, 4 VI 1944, Dreisbach (UMMZ) [study specimen #4597, slide mounted genitalia and wing, remainder pointed]; Mud Lake, 1 M, 1 VI 1950, Rogers (UMMZ); Oakland Co. 1 F, 13 VIII 1937, Rogers (UMMZ); Oscoda Co., 1 M, 24 VI 1947, Rogers (UMMZ); as in preceding but 1 M, 25 VI 1947; Pere Marquette State Forest – Dead Stream, N44° 18.317' W84° 34.820', 1208 ft, 4 MM, 1 F, 26 V 2009, J. Petersen (ISIC) [95% EtOH]; Rifle River State Recreation Area – Oyster Creek, N44° 24.598' W84° 02.135', 900 ft, 2 MM, 6 FF, 28 V 2009, J. Petersen (ISIC) [95% EtOH]; W. Branch Sturgeon River, 1 M, 27 V 1948, Leonards (UMMZ); Minnesota: Itasca State Park, 1500', 1 M, 29 VI 1970, Byers (KSU); New Hampshire: Bradford Bog, N43° 11.962' W72° 00.898', 975 ft., 3 MM, 1 F, 16 VI 2009, J. Petersen (ISIC) [95% EtOH]; Jefferson Notch, N44° 18.20' W71° 21.36', 2500 ft., 1 M, 27 VI 2009, M. Petersen (ISIC) [95% EtOH]; New Jersey: Hemlock Falls, 1 F, V, Unknown (ANSP); New York: Avalanche Trail, 1 F, 30 VII 1929, Melander (USNM); Batavia, 1 F, 18 VI 1913, Knight (CUIC); Browns Tract Bog, N 43.8° W 74.704722°, 1820 ft, 1 M, 29 VI 1980, McCabe (USNM); Browns Tract Bog, N43° 48.06' W74° 42.33', 1800 ft, 3 MM, 3 FF, 9 VI 2009, J. Petersen (ISIC) [95% EtOH]; as in preceding but 1 M, 20 VII 2009; Buell Mountain, 1300 ft., 2 MM, 15 VI 1916, Alexander (USNM); Buffalo, 1 M, VI 12 1910, Unknown (MCZ); Happy Valley Wildlife Management Area, N43° 25.278' W76° 00.248', 700 ft., 3 FF, 5 VI 2009, J. Petersen (ISIC) [95% EtOH]; Hurricane Mountain, 2000 ft., 2 MM, 12 VI 1927, Alexander (USNM); Lake Pleasant, 1750 ft., 1 M, 17 VI 1926, Alexander (USNM); as in preceding but 1 M, 1 F, 18 VI 1926 [male slide mounted abdomen and wing, remainder pointed]; as in preceding but
1 F, 20 VI 1926; Lancaster, 1 M, 1 F, 31 V 1908, MCV (CAS); Marcy Brook, 2200 ft, 1 M, 4 VII 1938, Alexander (USNM); Marcy Brook, N44.16992° W73.95504°, 2200 ft, 1 F, 19 VII 2009, M. Petersen (ISIC) [95% EtOH]; Massawepie Bog, N44° 14.12’ W74° 39.81’, 1550 ft., 2 MM, 1 F, 9 VI 2009, J. Petersen (ISIC) [95% EtOH]; Ox-Bow Lake, 1700 ft., 1 M, 25 VI 1925, Alexander (USNM); Ringwood Ponds, 1 M, 16 V 1981, Bickel (MCZ) [reared from pupae found in leaf litter, pupal exuviae pointed with adult specimen]; Sacandaga Park, 3 MM, 2 FF, 15 VI 1916, Alexander (USNM) [male specimen slide mounted genitalia and wing, remainder pointed]; Sapsucker Woods, N42.47777° W76.45423°, 975 ft., 1 M, 15 V 2009, J. Petersen (ISIC) [95% EtOH]; South Branch Grass River, N44° 14.511’ W74° 47.150’, 1500 ft., 1 F, 8 VI 2009, J. Petersen (ISIC); Tirrell Pond, 1 F, 19 VIII 1987, Brodo (FB); Woodsworth Lake, 2 MM, 7 VI 1961, Alexander (USNM); Pennsylvania: Bald Knob, 7.7km S. Central City along Rt 30, N40.0416° W78.788°, 2799 ft, 1 M, 29 VI 1996, Young (CMNH); Hazleton, 1 F, 23 V 1916, Dietz (ANSP); as in preceding but 1 M, 1 VI 1912; as in preceding but 1 F, 5 VII 1914; Philadelphia, 1 M, 11 VI 1891, Johnson (MCZ); Truemans, 2.2 km NW of town, N41.6325° W79.1530556°, 1706 ft, 1 F, 15 VI 1995, Young (CMNH) [UV light trap]; Vermont: Gifford Woods State Park, N43° 40.439’ W72° 48.676’, 1650 ft., 1 F, 12 VI 2009, J. Petersen (ISIC) [95% EtOH]; Green Mountain National Forest – Somerset Rd., N42° 54.727’ W72° 57.401’, 1800 ft, 1 M, 11 VI 2009, J. Petersen (ISIC) [95% EtOH]; Laurel Lake, 1 M, 1 F, 25 V 1975, G.K. Pratt (USNM); Moosalamoo, N43° 54.515’ W73° 01.088’, 1700 ft, 1 M, 2 FF, 12 VI 2009, J. Petersen (ISIC) [95% EtOH].

DISTRIBUTION. Found from Minnesota and Michigan eastward through Ontario, into New England and northeast into New Brunswick and Nova Scotia (Figure 3A). Populations are undocumented through northern Ohio, Indiana and Wisconsin, but likely survive in these areas given the appropriate habitat.

Ecological niche modeling produced a potential binomial distribution with relative accuracy based on the available data (AUC_Train = 0.8774, AUC_Test = 0.7523, test omission = 0.0438). The test omission for this species was the lowest of all models built, likely due to the high sample size (N=72). *Neophylidorea adusta* is the most widespread and abundant species of the genus, or at least the
most easily encountered due to accessibility to habitats in eastern North America compared with the western species. However, there may be some model overpredicting with this species as well. *Neophylidorea adusta* is unlikely to be found as far south as North and South Carolina. Crane flies have been extensively collection in this region and this species has not been recovered (Petersen et al. 2005). Likewise, the somewhat disjunct distribution to the north, matching the extent of the Canadian Shield is unlikely to provide suitable habitat. The single population in Minnesota from Itasca State Park is geographically disjunct from populations eastward. Despite attempts to collect specimens from the same locality and surrounding areas, *N. adusta* was not recovered any farther west than east-central Michigan. Appropriate habitat exists in northern Minnesota and Wisconsin, and future collecting should focus on these regions as they are likely undersampled.

**PHENOLOGY.** Individuals have been collected as early as May 20 (Mer Bleue, Ontario) and as late as September 6 (Brookline, Massachusetts). There is a single, extended flight period (Figure 4A). Even at a single location the flight period can be nearly six weeks (e.g., collected from Browns Tract Bog, New York on: June 9, June 29 and July 20). Julian days (avg=177.2, SD=29.6) are negatively correlated with latitude (avg = 43.5, SD = 4.2), although this effect is weak ($r^2 = 0.03$; $P=0.0429$).

**BIOLOGY.** *Neophylidorea adusta* is generally found flying or hanging from trees as adults near water. Habitat affinities include hemlock, beech, and swamp white oak forests often with large stands of ferns (e.g., cinnamon fern) and other herbaceous plant such as bunchberry dogwood and green false hellebore. Most often this species is collected from wet, swampy parts of forests (Alexander 1924), with nearly complete canopy cover. Adults can be found on the edges of open canopy bogs (e.g., Massawepie Bog, Adirondacks, New York) or within shrubs such as leatherleaf, bog Labrador tea or bog laurel, typical of sphagnum peat bogs. Altitudes vary widely, but are generally lower than the two species in western North America. *Neophylidorea adusta* has been collected as low as sea level (Maine) and up to 1000 m in Quebec.

In 2009, many populations of *N. adusta* were infected with a fungus, identified as Entomophthorales. Species of Entomophthorales have been noted in other Tipuloidea (Kramer 1980,
Hajek et al. 2003), but this is the first record of infection in Neophylidorea. All females with fungal infections were without eggs. In infected males, genitalia often became detached immediately upon handling. How prevalent this fungus is between years or how devastating the fungal infection can be to population dynamics are unknown. Typically, entire populations were infected (i.e., uninfected individuals were not encountered). The behavior of infected individuals differed from uninfected populations, but not in the typical manner. Individuals were always found alive, or barely so, and walking along the ground. This is contrary to the typical "elevation seeking" behaviors seen in many species infected with various fungal pathogens (Roy et al. 2006). One individual of Euphyllidorea platyphallus (Alexander) encountered in 2009 was infected with a morphologically similar fungus, but many other crane flies were collected and appeared healthy.

This species co-occurs with many other tipuloid flies. Sympatric species include: E. platyphallus, Prionolabis rufibasis (O.S.), Epiphragma fasciapenne (Say), and Trycyphona inconstans (O. S.). Species of Dolichopeza were also encountered, but not identified to species. Related species Bittacomorpha clavipes (Fabricius) and Ptychoptera quadrifasciata Say were also often collected along with N. adusta.

SYNONYMY. The type material for each synonomized species was available for study. The synonymy of E. terraenovae with N. adusta is based on geography (holotype from Newfoundland, Canada), size (body length: 11.5 mm; wing length: 12 mm; larger than co-occurring species with similar morphology, i.e., E. similis and E. platyphallus) and wing morphology (relatively long Rs compared to E. similis and E. platyphallus). Alexander noted the similarities between N. adusta and E. paeneadusta in the original description of the later species. The differences he saw between these species were based on wing and thorax coloration, which was shown through multivariate analysis in Chapter 3 to have little correspondence to the definition of species in this group. Another character Alexander considered as species-defining was the shape of the ventral paramere (referred to as 'basal lobes of the sternum' by Alexander; "narrow blade" in E. paeneadusta and "subtriangular blade" in N. adusta). The genitalia of the E. paeneadusta holotype is slide mounted, as is typical for Alexander types. In this instance, the aedeagal complex was in part misshapen through the mounting
process. Therefore, the differences in ventral paramere shape between this type specimen and other specimens of *N. adusta* can be attributed to a lateral view of the structure rather than the more typical ventral view.

**REMARKS.** *Neophylidorea adusta* is morphologically and genetically closely related to *N. columbiana* (Chapter 3). Distinguishing features include numerous cloudy patches on wings and geographic distribution. These are considered separate species for these reasons despite paraphyly in both the nuclear or mitochondrial gene trees (Chapter 3).

**Additional Material Examined**

The following specimens were examined and reliably identified as *Neophylidorea*. However, they lacked key defining characters which generally allow for species-level identification. The majority are females or specimens lacking genitalia. Discriminate function analysis was used where possible, but resulted in low probability for any single species (Prob. <0.95).

**CANADA.** British Columbia: Forbidden Plateau, 1 F, 16 VIII 1950, Guppy (UMMZ).

**UNITED STATES.** Alaska: Eagle River, SE Alaska, 1 F, 14 VI 1952, F. (USNM); California: Cuyamaca State Park, 1 F, 28 VI 1963, Alexander (USNM) [one additional specimen lacking genitalia]; Hatchet Pass, 4200 ft., 2 FF, 12 VIII 1948, Alexander (USNM); Sonoma Co., 2 FF, 26 VI 1914, Unknown (USNM); Oregon: Little Cultus Lake, Deschutes National Forest, N43.8004° W121.8781°, 4800 ft., 1 F, 27 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Olive Lake, Umatilla National Forest, N44.7830° W118.5955°, 6100 ft., 5 FF, 27 VI 2007, J. Davis and M. Petersen (ISIC) [95% EtOH]; Panther Creek, Willamette Valley, 14 VII 1948, Fender (USNM) [lacks genitalia]; Utah: Wellsville, Cache Co., 4 IX 1942, Knowlton (USNM) [lacks genitalia]; Washington: Longmire Springs, Mt. Rainier, 2 FF, 10 VI 1917, Dyar (USNM); Pullman, 1 F, IX, Unknown (USNM) [perhaps flavapila syntype, but not definitive]; Wyoming: Emerald Pool, Yellowstone National Park, Black Sand Geyser Basin, 7275 ft., 29 VI 1941, Alexander (USNM) [slide mounted wing, remainder pointed but lacks genitalia]; Roosevelt Station, Yellowstone National Park, 1 F, 5 VII 1923, Melander (USNM).
Doubtful Species

*Limnophila (Phylidorea) aequiatra* Alexander, 1949b: 314 [original designation].


**REMARKS.** This species was described from a female only, and although it clearly belongs to *Neophylidorea*, it lacks the group defining characters to associate with either *N. columbiana* or *N. flavapila*. The wing of the holotype specimen was too warped to analyze using discriminate function analysis. Attempts were made in 2008 to collect specimens from Galena Camp and Hood River Meadows in late July, but no populations of *N. flavapila* or *N. columbiana* were recovered.

**Taxonomic Key to the Adult Males of Neophylidorea**

1. Median lobe of the ninth tergite protruding from the surface as either bulbous (length equal to width; Figure 5E) or elongate (length greater than width; Figure 1E).........................2

2. Median lobe of the ninth tergite flush with the surface or nearly so (can also be slightly concave ventrally on some dried specimens, but still flattened; Figure 8E).........................3

3. Median lobe of the ninth tergite a finger-like projection at a 35-degree angle from the surface, longer than wide and rounded at the apex (Figure 1E). Rs exceptionally short. Ventral parameres elongate and rounded apically (Figure 9B).............*Neophylidorea caudifera*

   Median lobe of the ninth tergite bulbous (Figure 5E). Ventral parameres hooked apically (Figure 9D).................................................................*Neophylidorea flavapila*

4. Central aedeagal filament bent at 90-degrees apically (Figure 7B)..........................4

5. Central aedeagal filament straight but slightly fluted apically (Figure 8)......................5
4. Ventral parameres deeply hooked apically with toothed flat surface preceding hook (Figure 9C).................. Neophylidorea vanronea sp. nov.

Ventral parameres with shallow, lateral spine (Figure 9A)........... Neophylidorea neadusta

5. Distributed east of the Mississippi River. Shading on wing apex, along cord, at base of Rs and/or along $R_2,3,4$......................... Neophylidorea adusta

Distributed west of the Mississippi River and usually lacks shading on wing .................

................................................................. Neophylidorea columbiana

**Phylogenetics**

Fourteen characters were developed based on morphological variability between species of *Neophylidorea* as well as the known synapomorphies of the genus. The following is a description of the characters and associated character states:

01. **Aedeagus. Length of filaments** (0) all long branches (1) short branches. This character in part defines the ingroup (*Neophylidorea*). *Euphylidorea similis* and related species are the only other known group of “Limnophilinae” species to bear a trifid aedeagus. The remaining outgroups have a singular aedeagal opening.

02. **Aedeagus. Median filament apex** (0) strongly bent at 90-degrees apically (1) straight. *Neophylidorea adusta, N. columbiana* and *N. caudifera* all have the central aedeagus that is straight or slightly fluted apically (Figures 1 and 8). All remaining species have a sharp bend of the central aedeagal filament (Figures 5 and 7, Figure 2F from Chapter 2)

03. **Aedeagus. Basal condition** (0) curved dorsally (1) straight. This character is another synapomorphy of *Neophylidorea*. Although some of the outgroup taxa have a trifid aedeagus (*E. similis*), all three filaments are straight from the base to the apex. *Neophylidorea* species minimally have the central aedeagal filament (sometimes all three filaments) curved dorsally before straightening out apically.

04. **Ventral paramere. Deeply divided** (0) absent (1) present. Like the first character, this is included to separate the outgroups from the ingroup. Likely closely related species of *Euphylidorea*
usually have a deeply divided ventral paramere. The homology of the ventral parameres has yet to be well understood, thus making scoring for *Epiphragma fasciapenne* difficult at this time.

05. **Ninth tergite. Median lobe** (0) flat (1) raised. All species of *Neophylidorea* possess a median lobe on the ninth tergite, but the shape varies among species. This character is referring to the 3-dimensional aspect of the median lobe in *N. flavapila* (Figure 5E) and *N. caudifera* (Figure 1E) in comparison to the flattened median lobe of other species.

06. **Ninth tergite. Median lobe invagination** (0) present (1) absent. Alexander often commented on the invagination of the median lobe and the membranous area at the base of the invagination that it accompanies. Therefore this character is also used in the present analysis and is informative in delimiting species.

07. **Ninth tergite. Median lobe setation** (0) sparse or absent (1) dense. This character is obvious to the observer as the setae are stout and abundant or absent from the median lobe.

08. **Ninth tergite. Lateral process of the median lobe** (0) reduced or absent (1) prominent. This character is found throughout other taxa (Brodo 1987) and has been used previously in crane fly phylogenetics.

09. **Ventral gonostylus. Apex melanization** (0) present (1) absent. The ventral gonostylus of *Neophylidorea* is fleshy and, although hooked at the end, does not bear an acutely melanized bifid tip like species of *Euphylidorea*.

10. **Ventral paramere. Apex** (0) hooked (1) straight. Two species of *Neophylidorea* possess a deeply (*N. vanronea* sp. nov.) or shallowly (*N. flavapila*) hooked apex of the ventral paramere (Figure 9C-D).

11. **Gonoxite. Knob at base of dorsal gonostylus** (0) absent (1) present. A few species have a knob or bulb near the apex of the gonoxite, just basal to the connection of the dorsal gonostylus with the gonoxite (Figures 1A, 5A).

12. **Gonoxite. Inner surface invagination** (0) absent (1) present. In all species of *Neophylidorea* the inner surface of the gonoxite is fleshier than the outer surface. *Neophylidorea*
*adusta* and *N. columbiana* have an invagination of the hardened surface into the fleshy portion near the apex of the gonocoxite (Figure 8A).

13. Wing. *Apical clouding* (0) absent (1) present. An analysis of various morphological characters demonstrated that this character is useful in distinguishing some species from others (e.g., *N. columbiana* from *N. adusta*; Chapter 3).

14. Wing. *Clouding along cord* (0) absent (1) present. As with the previous character, this may provide some resolution among species.

For a direct comparison, scoring of the 22 ingroup taxa was based on the same specimens used in the molecular analysis. Individuals within a species were scored identically for each character, with one exception (Table 3). The population of *Neophylidorea columbiana* collected from Wyoming (*N. columbiana* – 3) in the far eastern range (Figure 6A) has wing clouding at the apex [character 13(1)] and along the cord [character 14(1)]. Three outgroup taxa were scored for each character, although these were often inapplicable due to lack of homologous structures.

Molecular sequences produced 2196 base pairs of COI and COII for 22 specimens including two outgroups (*Epiphragma fasciapenne* and *Euphylidorea platyphallus*) and 1056 base pairs of CAD for the same ingroup individuals and two outgroups (*Epiphragma fasciapenne* and *Euphylidorea similis*). Phylogenetic analyses produced somewhat similar gene trees for mitochondrial versus nuclear datasets (Figure 10).

The mitochondrial gene tree indicated strong support for the most ancestral taxa being *N. caudifera*, which is sister to a paraphyletic *N. flavapila* clade. The population of *N. flavapila* from the Sierra Nevada’s in California consistently indicates some level of paraphyly within this taxa (Chapter 3). *Neophylidorea adusta* and *N. columbiana* form a well-defined monophyletic clade (posterior probability (pp) = 1.0; referred to as *adusta-columbiana* clade), although the species themselves are paraphyletic. *Neophylidorea columbiana* is especially paraphyletic forming only a single monophyletic clade (populations 4, 5 and 6) and lacking any geographic structure. *Neophylidorea adusta* is likewise paraphyletic, but only with a single taxon (population 8) grouping with a population
of *N. columbiana* from Idaho (populations 1 and 2). The remainder of the *N. adusta* taxa form a well supported clade (populations 1, 2, 3, 4, 5, 6 and 7).

The relationships among species are for the most part the same for the nuclear gene tree. *Neophylidorea caudifera* is again well supported as the most ancestral species of the genus (pp = 1.0). The biggest difference between the gene trees is that here *N. flavapila* forms a monophyletic clade (pp = 1.0). Sister to *N. flavapila* again is a well supported *adusta - columbiana* clade (pp = 1.0).

Another striking difference is the monophyletic clade formed by some *N. columbiana* taxa (populations 4, 5, 6, 7 and 8; pp = 0.95). These taxa are somewhat geographically structured as they are all located in the western range of the species (California, Oregon and Washington). However, the species itself is again not monophyletic. Three *N. columbiana* taxa (populations 1, 2 and 3) form a monophyletic clade (pp = 0.96) along with several widely geographically separated *N. adusta* taxa (populations 6 and 8). The remaining *N. adusta* taxa form the most derived clade (populations 1, 2, 3, 4, 5, and 7). The result of a combined mitochondrial and nuclear gene tree produced a topology identical to that of the mitochondrial gene tree and is therefore not illustrated here.

The combined molecular and morphological parsimony analysis included 25 taxa including three outgroups. The traditional search produced 5 equally parsimonious trees (MPT) with a length of 1310 steps. The consensus tree (Figure 11) built from all five MPT had a retention index (RI) of 0.78 and a consistency index (CI) of 0.83. Bootstrap support (bs) was relatively strong for the most ancestral clades, but weak for more derived taxa. As with the mitochondrial gene tree, *N. flavapila* is paraphyletic with respect to the California population. *Neophylidorea vanronea* sp. nov. forms a clade (bs = 80%) with *N. neadusta* as sister taxa (bs = 53%) to the *adusta-columbiana* clade (bs = 68%).

As with the gene trees, this combined analysis produced a variety of paraphyletic groupings between *N. adusta* and *N. columbiana*, but again these two species form a monophyletic *adusta-columbiana* clade. One well supported clade (bs = 81%) forms the basis for the paraphyly associated with both *N. adusta* and *N. columbiana* and consisted of a population of *N. columbiana* from Idaho (1 and 2) and *N. adusta* from Maine.
Discussion

Prior to this work, many museum specimens were unidentified or misidentified, and few of the newly collected specimens could be identified to species with any confidence. Because of the extensive efforts to delimit species of Neophylidorea (Chapter 3) and the redescriptions, detailed illustrations and taxonomic key included in the present work, these species are now readily identifiable. Some aspects of the biology and ecology are better understood such as habitat affinities, flight periods, elevation, and geographic distribution. As typical of crane flies, the majority of our knowledge rests in the adult males, with little information regarding morphological differentiation among females and immature life stages.

Despite the lack of support within species for some phylogenetic analyses, especially with respect to N. adusta and N. columbiana, the general evolutionary relationships among species were recovered and congruent among analyses (Figure 12). All analyses (mitochondrial, nuclear, and molecular+morphological) produced a phylogeny with N. adusta and N. columbiana as the most derived sister species. The combined morphological and molecular analysis indicates that N. neadusta is sister to the adusta-columbiana clade, although there is little support for this relationship. There is more support for a clade including N. vanronea (N. neadusta(adusta-columbiana)). Neophylidorea flavapila is clearly sister to the larger clade described previously. Lastly, N. caudifera is sister to all other species of Neophylidorea.

Clearly, there are some paraphyletic species, especially regarding N. columbiana and N. adusta. This is not surprising given the results of the methods used to delimit species (Chapter 3). Paraphyly is also not surprising given the known lack of concordance between gene trees and species trees (Pamilo and Nee 1988, Maddison 1997, Nichols 2001). However, ecologically these two taxa are quite distinct (Chapter 3). Morphologically (based on wing geometric morphometrics and character-based tests of morphological features) N. adusta and N. columbiana are distinct. Therefore, given these various sources of data suggesting near separation they are considered separate species.
From a morphological phylogenetic perspective, some aspects of the evolution of character states can be discussed. The flattened median dorsal lobe on the ninth tergite [character 5(0)], the median invagination of the median lobe [character 6(0)], and prominent lateral processes [character 8(1)] are synapomorphies for the clade formed by *N. vanronea* sp. nov., *N. neadusta*, *N. adusta* and *N. columbiana*. The absence of a knob at the base of the inner gonostylus on the gonocoxite [character 11(0)] is a synapomorphy for the *N. neadusta*, *N. adusta* and *N. columbiana* clade. Many characters used in this analysis included aspects of male genitalia, which are not any more or less likely to track evolutionary history as compared with non-genitalic characters (Song and Bucheli 2010). Although we were unable to include characters from immature life stages in the phylogenetic analysis, morphology of immatures would have likely provided little resolution at the species-level (Meier and Lim 2009).

Two of the six recognized *Neophylidorea* species are known from a single locality and only one or two specimens. Despite attempts to sample at and around the type localities for both species, no recent collections of these species have been made. Although identifying these two taxa as valid species is somewhat contrary to the premise of the previous attempts to quantitatively delimit species using a broad spectrum of data (Chapter 3), those results indicated that the original characters used to delimit species can be used as evidence of distinct species (e.g., ventral paramere shape). Therefore, because these two species also differ morphologically in the ventral paramere shape, we concluded that these two species are also likely distinct. Whether or not additional populations of these species will be recovered and what affect that will have on the taxonomy and phylogenetic relationships among species remains to be seen. These species hypotheses should be revised given additional data.

Other researchers have not synonymized such a high percentage of species described by Alexander. Petersen (2008) had a low percentage of synonymy in his revision with only 3% of the species synonymized. Gelhaus in his revision of *Tipula* (*Eremotipula*) synonymized 14% of species in the group (2005). Brodo synonymized 43% of species in her revision of *Prionocera* (1987). The current revision is the highest with a rate of approximately 63%. Almost all of these revisions were
higher than the overall error rate in Tipuloidea (~10%, Oosterbroek 2010). This revision was the result of careful investigation using multiple methods and incorporating hundreds of specimens to ensure the most accurate representation of species-level diversity possible.

Although this research has greatly expanded our knowledge of the genus *Neophylidorea*, there is still much that can be learned. Collections are sparse from northern latitudes, especially in western North America. Future research should focus on collecting and associating larvae. The ability to identify larvae by morphology alone is unclear, but this work demonstrates that molecular techniques are a useful tool for associations with the ability to retain the original specimen for future reference. Ideally, *Neophylidorea* should be placed in a large phylogenetic context, but this will not be possible until there is a better understanding of “Limnophilinae” phylogenetics.

**References**


Alexander, C.P. (1920a) Scientific results of the Katmai expedition of the National Geographic Society. The crane-flies (Tipulidae, Diptera). *Ohio Journal of Science* 20, 193-203.


**Acknowledgements**

Sigitas Podenas was helpful in translating some of Savchenko’s work. Matt Petersen, Fenja Brodo and Jon Gelhaus provided support regarding many aspects of crane fly systematics. Funding was provided by the Biosystematics Travel Grant - Iowa State University, Henry and Sylvia
Richardson Award, Graduate Student Award from the Society of Systematic Biologists and the Ernst Mayer Travel Grant in Animal Systematics. All material was collected under appropriate collecting permits: Parks Canada permit number GLA-2008-1733, and U.S. National Park Service study number ACAD-00180.
List of Figures

Figure 1. Male genitalia of *N. caudifera*. A. dorsal view of hypopygium, B. dorsal view of male aedeagal complex, C. ventral view of hypopygium, D. ventral view of aedeagal complex, and E. lateral view of hypopygium. Abbreviations: 9s = ninth sternite, 9t = ninth tergite, aed = aedeagus, d gonst = dorsal gonostylus, dpa = dorsal paramere, ej ap = ejaculatory apodeme, goncx = gonocoxite, ib = interbase, ml = median lobe, sp = sperm pump, v gonst = ventral gonostylus, vpa = ventral paramere.

Figure 2. Wing morphology. Abbreviations: Veins - A₁, A₂ = branches of anal veins; C = Costa; CuA = Anterior branch of Cubitus; CuA₁, CuA₂ = Anterior branches of Cubitus; M = Media; R = Radius; R₁+₂: Anterior branch of Radius plus R₂ posterior branch of Radius; R₂ = posterior branch of Radius connecting Radial sector to Radius; R₃, R₄, R₅ = Posterior branches of Radius; Rs = Radial sector; Sc = Subcosta. Cells - a₁, a₁ = Anal; bm = Basal Medial; br = Basal Radial; c = Costal; cua₁ = Anterior Cubital; cup = Posterior Cubital; dm = Discal Medial; m₁, m₂, m₃ = Medial; r₁, r₂, r₃, r₄, r₅ = Radial; sc = Subcostal.

Figure 3. Distribution of eastern Nearctic *Neophylidorea* species. A. *N. adusta*, B. *N. caudifera*.

Figure 4. Frequency distribution of *Neophylidorea* specimen abundance across Julian days. Julian days (i.e., January 1 = 1, December 31 = 365) are plotted by the number of specimens for each 5 day period over the flight period. Data was taken from all available specimens and includes one value for each collection irrespective of the number of specimens collected at a given time and location. A) *N. adusta*, N=183; B) *N. caudifera*, N=32; C) *N. columbiana*, N=163; D) *N. flavapila*, N=155.
Figure 5. Male genitalia of *N. flavapila*. A. dorsal view of hypopygium, B. dorsal view of male aedeagal complex, C. ventral view of hypopygium, D. ventral view of aedeagal complex, and E. lateral view of hypopygium. Abbreviations: 9s = ninth sternite, 9t = ninth tergite, aed = aedeagus, d gonst = dorsal gonostylus, dpa = dorsal paramere, dpr = dorsal process, ej ap = ejaculatory apodeme, goncx = gonocoxite, ib = interbase, ml = median lobe, sp = sperm pump, v gonst = ventral gonostylus, vpa = ventral paramere.

Figure 6. Distribution of western Nearctic *Neophylidorea* species. A. *N. columbiana*, B. *N. flavapila*.

Figure 7. Male genitalia of *N. vanronea* sp. nov. and *N. neadusta*. A. Dorsal view of holotype, *N. vanronea* sp. nov. specimen, B. dorsal view of holotype, *N. neadusta* specimen. Abbreviations: 9t = ninth tergite, aed = aedeagus, d gonst = dorsal gonostylus, dpr = dorsal process, ej ap = ejaculatory apodeme, goncx = gonocoxite, ib = interbase, ml = median lobe, sp = sperm pump, v gonst = ventral gonostylus, vpa = ventral paramere.

Figure 8. Male genitalia of *N. columbiana*. A. dorsal view of hypopygium, B. dorsal view of male aedeagal complex, C. ventral view of hypopygium, D. ventral view of aedeagal complex, and E. lateral view of hypopygium. Abbreviations: 9s = ninth sternite, 9t = ninth tergite, aed = aedeagus, d gonst = dorsal gonostylus, dpa = dorsal paramere, dpr = dorsal process, ej ap = ejaculatory apodeme, goncx = gonocoxite, ib = interbase, ml = median lobe, sp = sperm pump, v gonst = ventral gonostylus, vpa = ventral paramere.

Figure 9. Ventral paramere shape of *Neophylidorea* species. A. *N. neadusta*, B. *N. caudifera*, C. *N. vanronea* sp. nov., D. *N. flavapila*, E. one extreme of the morphological variation encompassed within *N. columbiana*, F. opposite extreme of the variation encompassed within *N. columbiana*, and typical morphology of *N. adusta*. 
Figure 10. Molecular phylogeny of the genus *Neophylidorea* based on Bayesian analysis. The left phylogeny is a gene tree based on the two mitochondrial genes, COI and COII. The right phylogeny is based on a nuclear gene, CAD. The common scale of 0.5% divergence is included. Posterior probabilities are displayed at branch nodes where applicable (>0.5).

Figure 11. Combined molecular and morphological phylogeny of *Neophylidorea* based on parsimony analysis. Bootstrap values are shown for nodes with support >50%.

Figure 12. Pictorial diagram of hypothesized evolutionary relationships among *Neophylidorea* species. Terminal taxa are species bordered by the dark lines. Individuals are indicated by thin lines. Incomplete lineage sorting or historical hybridization between *N. adusta* and *N. columbiana* is marked with an open circle.
Figure 1
Figure 3

Legend
Species
- N. adusta
- Political Boundaries

N. adusta potential distribution
<Probability>
- 0 - 0.13
- >0.13 - 1.0

Legend
Species
- N. caudifera
- Political Boundaries

N. caudifera potential distribution
<Probability>
- 0 - 0.13
- >0.13 - 1.0
Figure 4

A

B

C

D
Figure 5

A

B

C

D

E
Figure 6

Legend
species
○ N. columbiana
—— Political Boundaries

N. columbiana potential distribution
<Probability>

0 - 0.16
0.16 - 1.0

A

Legend
species
○ N. flavapila
—— Political Boundaries

N. flavapila potential distribution
<Probability>

0 - 0.10
0.10 - 1.0

B

Km
0 300 600 1,200 1,800 2,400
Figure 8

A

B

0.05mm

C

D

E
Figure 10

N. adusta - 1
N. adusta - 2
N. adusta - 3
N. adusta - 4
N. adusta - 5
N. adusta - 6
N. adusta - 7
N. columbiana - 1
N. columbiana - 2
N. adusta - 8
N. columbiana - 3
N. columbiana - 4
N. columbiana - 5
N. columbiana - 6
N. columbiana - 7
N. columbiana - 8
N. flavapila - 1
N. flavapila - 2
N. flavapila - 3
N. caudifera - 1
Figure 11

N. adusta - 1
N. adusta - 2
N. adusta - 3
N. adusta - 4
N. adusta - 5
N. adusta - 6
N. adusta - 7
N. adusta - 8
N. columbiana - 1
N. columbiana - 2
N. columbiana - 3
N. columbiana - 4
N. columbiana - 5
N. columbiana - 6
N. columbiana - 7
N. columbiana - 8
N. neadusta
N. vanronea
N. flavapila - 1
N. flavapila - 2
N. flavapila - 3
N. caudifera - 1
Figure 12
Table 1. Environmental variables used in ecological niche modeling of *Neophylidorea* species.

Variables included in the final model for each species are indicated with an “x” along with a description of each variable.

<table>
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<th>flavapila</th>
<th>caudifera</th>
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<td>Annual Mean Temperature</td>
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<td>Bio2</td>
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<td></td>
<td>x</td>
<td>x</td>
<td>Mean Diurnal Range</td>
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<td>Bio3</td>
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<td></td>
<td>x</td>
<td>Isothermality</td>
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<td>x</td>
<td>x</td>
<td></td>
<td>Min Temperature of Coldest Month</td>
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<td>Bio7</td>
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<td></td>
<td></td>
<td></td>
<td>Temperature Annual Range</td>
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<td>x</td>
<td>Mean Temperature of Wettest Quarter</td>
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<td>Bio9</td>
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<td>Mean Temperature of Driest Quarter</td>
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<tr>
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<td>Mean Temperature of Warmest Quarter</td>
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<td></td>
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<td></td>
<td>x</td>
<td>Annual Precipitation</td>
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</tr>
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<td>x</td>
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</tr>
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<td>x</td>
<td>Precipitation of Coldest Quarter</td>
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<td>CTI</td>
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<td>x</td>
<td></td>
<td>x</td>
<td>Compound Topographic Index</td>
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Table 2. Taxa used in molecular and morphological phylogenetic analyses. Populations collected in 2007-2009 from across the range of the genus.

<table>
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<th>Description</th>
<th>Latitude</th>
<th>Longitude</th>
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<tr>
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<td>USA, ME, York Co.</td>
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<tr>
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<td>Dollar Lake Provincial Park</td>
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<tr>
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<td>Jackson, Conservation Research Fen</td>
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Table 3. Matrix of characters and alternate states. Detailed locality descriptions of *Neophylidorea* populations are included in Table 2. Inapplicable character states are indicated by ‘-’. See the text for a description of each character and alternate states.

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CHAPTER FIVE: GENERAL CONCLUSIONS

This dissertation tracked the progress of defining a monophyletic group of insects, followed by a detailed, integrated approach to delimiting species of a genus and formally revising the species to include synonymized species, redescriptions and illustrations, and the description of a new species discovered during the course of this research. The result is a better understanding of the ecology and evolutionary biology of a group of flies. Prior to this research, little was known about the biology of this group or the species within. Through collecting and identifying populations, and assessing various aspects of the natural history of Neophylidorea species, I added considerable information to our knowledge of the biology of this group. New methods were employed and integrated to form a better understanding of the limits of species. The criteria used to delimit species included aspects of molecular biology, morphology, and ecology. Through integration of these methods, I identified the potential false-positives and false-negatives produced by various methods, therefore providing support for some criteria over others. By utilizing a number of different criteria, I proved that the originally described diversity in this group was overestimated by more than 60%. From a practical standpoint, this research provided the tools necessary for other biologists to identify species of Neophylidorea and forms a stable taxonomic structure upon which future research can be built.

With regards to the research interests in systematic biology, this dissertation addressed the first five of the “seven great questions in systematic biology” (Cracraft 2002). The first question addressed here is “What is a species?”. Chapter 3 discusses in depth the process of delimiting species including four quantitative methods encompassing morphology, molecular, and ecological datasets. I approached the second question, “How many species are there?” by proposing a new species hypothesis in Chapter 2 and revising long standing hypotheses of species in Chapter 4. The analyses in Chapter 3, 10 species were synonymized, thus decreasing the total number of valid species of crane flies. Through a phylogenetic analyses using morphology and molecular data in Chapter 4, I addressed the third question: “What is the Tree of Life?”. I proposed a combined morphological and molecular phylogeny of the genus Neophylidorea. This is an area where much more work is necessary to place Neophylidorea in a larger phylogenetic context. Morphological
character transformations were analyzed by parsimony analysis in Chapter 4, partially answering the question, “What has been the history of character transformation?”. Lastly, by including ecological niche modeling in both Chapters 3 and 4, the distribution of species was modeled which addressed the fifth question: “Where are Earth's species distributed?”. These five major questions, and including more practical implications such as a taxonomic key to species, this dissertation contributes significantly to the field of systematics.

In addition, the reach of this dissertation is far beyond systematic biology and the broad impacts of this project include two components. The first is a general greater understanding of ecology and evolutionary biology. This project bridges the gap of the sometimes disparate fields of evolution, ecology, systematics and conservation biology. I provide a synergism between these fields by taking into consideration the many different biological aspects affecting species divergence. The conservation implications of aspects such as climate change or habitat loss on species survival cannot be fully understood until the processes which lead to species formation and continued survival are resolved. This study aims to understand the phylogeography of crane flies that inhabit wet montane meadows, which may be sensitive to climate change. Ecological niche modeling provided insight into abiotic variables that determine each species distribution, thus adding to our ecological knowledge of these fragile wetland ecosystems and the crane flies that inhabit them. I discovered a previously unknown phenomenon of fungal infection in *N. adusta*, which may have implications for conservation biology as it seems to severely impact population dynamics. The methods I used to understand the limits of species incorporate aspects of genetic evolution, morphological evolution and ecology. These varied considerations of species limits told approximately the same story with regards to the number of *Neophylidorea* species.

The second impact is a better understanding of crane fly biology at multiple taxonomic levels, from the gene to higher level phylogenetics. One novel aim of this project is the use of molecular data to resolve crane fly species limits and phylogenetics. At the beginning of this project, no published works of molecular phylogenetics for crane flies existed. Since then, two papers have been published (Nitta and O'Grady 2008; Petersen et al. 2010) on generic and family-level crane fly
phylogenetics and one Master’s thesis attempting to resolve subfamilial relationships (Ahnonen 2008). When first attempting to use “universal” primers for gene sequencing, the primer match may not be specific enough to properly amplify a gene fragment. My work towards developing new primers for cytochrome oxidase (COI and COII) and rudimentary (CAD) will help others in the field by decreasing troubleshooting time. Lastly, I used a novel application of “DNA barcoding” to identify female and larval specimens that would typically not be identifiable to species using traditional morphological characters. My approach to resolving the true diversity of crane flies through systematic revisionary work at the genus level has ultimate implications for higher level phylogenetics.

Rarely are the methods used to identify species accounted for during the revision process. Seemingly discrete morphological characters may be discussed, but with little regard to the likelihood of character fixation or sample size (Wiens and Servedio 2000). Here, I included multiple criteria nested within each data type to repeatedly test four species hypotheses based on gross morphology. This research paves the way for others by demonstrating how these methods can be tested in concert and illustrates the need for multiple methods from the apparent incongruence found.

**Direction of Future Research**

Open access to datasets is important to exchanging data rapidly and scientific progression, particularly for taxonomy (Penev et al. 2008). There is clearly a great need and desire for this type of open access information, especially with regard to insects (Clarke 2002). But these networks rely on taxonomists and biologists in general to provide the data (Agosti 2003). After the manuscripts from this dissertation have been accepted for publication, I will submit my datasets to various online sources such as the Global Biodiversity Informatics Facility (GBIF, http://data.gbif.org), a portal for distributional data. I will also submit the morphological matrix used to assess phylogenetics in Chapter 4 to MorphoBank (http://www.morphobank.org/) which stores data on the tree of life. Likewise, the molecular data will all be submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as part of the publication process. The COI data will also be submitted to the Barcode of Life Data

This project could certainly continue beyond the current analyses, especially given additional data. More populations of the rather rare species including the newly described species *Neophylidorea vanronea*, *N. neadusta* and even *N. caudifera* may improve the taxonomy of these species. Further sampling of this group throughout the entire range including areas of Colorado, Utah, northern British Columbia and into Alaska would give a broader understanding of the morphological and genetic diversity. Additional sampling of difficult taxa such as *N. columbiana* and *N. adusta* would add data to each of the datasets (ecological, morphological, molecular) used to define species limits and may provide additional insight into the potential mechanisms of speciation in these recently diverged groups.

Additional revisionary work of *Euphylidorea*, especially the remaining species that have a trifid aedeagus is still necessary. A broader context of “Limnophilinae” evolution, and in turn because the group is paraphyletic, a better understanding of crane fly phylogenetics in general.

References


ACKNOWLEDGEMENTS

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There is no way I could have completed this body of work without my dear husband, friend, mentor, and colleague, Matt Petersen. Not only did he travel with me to collect, advised me of crane fly biology, and read drafts of papers but also cooked meals when I was too distracted and supported me emotionally through this roller coaster.

Thanks to Irby Lovette and all the members of the Fuller Evolutionary Biology Lab at Cornell University – Lab of Ornithology. Irby took a leap of faith allowing me to work in his lab, supporting me through finding my way in evolutionary genetics. The unconditional support I received from the members of the Fuller Lab was amazing. I made connections there I will forever be grateful for.

There were many other people that made the logistics of this work possible. I had help from many museum staff including Holly Williams and Mark O’Brien, among others. Fellow crane fly researchers provided specimens as well as insight into biology. Fenja.Brodo welcomed me into her home, went collecting and told stories of Charles Alexander, all of which gave me a great appreciation for crane fly taxonomists. Jon Gelhaus also provided crane fly insight at various times and was a great host. Mike Collyer was always willing to help with morphometrics analyses.

My parents were troopers throughout this research providing all kinds of support. Both of my parents on several occasions helped with collecting. My dad especially endured a long and grueling collecting trip with me.

Many other friends supported my efforts along the way. Wendy and Nina provided scientific and emotional support. Jen let me camp out at her house for weeks on end.

It takes a village. Thanks!