ECOLOGY AND PHYLOGENY OF THE
BITING-MIDGE GENUS *CULICOIDES*
(DIPTERA: CERATOPOGONIDAE)

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ABSTRACT

Flies of the genus *Culicoides* are small, biting midges in the family Ceratopogonidae. These flies are of medical, veterinary, and economic importance because their blood-feeding behavior can cause stress to hosts and transmit disease agents. Despite the importance of these flies, little is known about their biology, especially the ecology of the immature stages and the phylogenetic relationships among taxa. The objectives of my study were to address these two areas of *Culicoides* biology.

Larval *Culicoides* were collected from aquatic habitats in four ecoregions of South Carolina, USA. Eleven ecological variables were recorded for each sample. Larvae were identified by amplifying and sequencing a portion of the COI gene by PCR and performing a BLAST search of an adult COI database. BLAST identifications were confirmed with morphological descriptions. Multiple logistic regression of the ecological variables was conducted on the presence-absence of larval taxa. Eleven species, 1 species complex, and 3 unidentified morphospecies of *Culicoides* were identified. Logistic regression yielded predictive models for *C. furens* (Poey), 1853, and *C. hollensis* (Melander and Brues), 1903. *Culicoides haematopotus* Malloch, 1915, was composed of five genetic clusters and two ecological groups, one present in shallow, aquatic habitats associated with hardwood forests and the other with shallow, aquatic habitats in the coast plains ecoregion, indicating a probable species complex. Two larval taxa with distinct morphologies were linked to *C. stellifer* (Coquillett), 1901, indicating another probable species complex.

Examination of *Culicoides* species for potential synapomorphies of the genus yielded two smooth, cuticular structures (scutal areolae) on the scutum just anterior to the scutellum. Scutal areolae were documented in males and females of seven genera of Ceratopogonidae. The scutal areolae were a synapomorphy of the family, with independent losses in the Forcipomyiinae+Dasyheleinae and the Ceratopogonini. In *Culicoides* and *Paradasyhelea,* the scutal areolae were modified into raised nodules, supporting a sister group relationship of these taxa. No pores, muscles, or nerves were associated with the scutal areolae, but the structures had light-reflecting properties, indicating a possible role in intraspecific communication.

The subgeneric classification of *Culicoides* was assessed using cladistic analysis. Morphological characters were extracted from the literature and used in a maximum parsimony analysis of the 13 subgenera and 7 species groups of Nearctic *Culicoides*. Five subgenera and one species group of *Culicoides* were monophyletic. Three subgenera were polyphyletic and no supporting synapomorphies were found for 10 subgeneric groups. A clade of the subgenera ((*Avaritia*+*Hoffmania*)+*Culicoides*) was inferred from the morphological analysis and confirmed by a maximum likelihood analysis of a fragment of the COI gene. Maximum likelihood analysis of an unresolved polytomy, using COI, did not result in improved resolution of the morphological tree, but indicated a species complex for *C. stellifer*, supporting results from the larval ecology study.

DEDICATION

I dedicate this dissertation to my wife Lane and my son Trent. Thank you for your encouragement, support, and patience while I pursued my dream.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Introduction

Culicoides are small flies in the family Ceratopogonidae. In North America, they are commonly referred to as biting midges, no-see-ums, or punkies. The genus is the most diverse genus of the family Ceratopogonidae, with more than 20% of the species in the family (Borkent 2012a). They are cosmopolitan in distribution occurring on every continent except Antarctica and at elevations up to 4200 m (Borkent 2004). *Culicoides* is the main pest genus of the family because females of many species feed on vertebrate blood for maturation of eggs. This blood-feeding behavior can lead to transmission of disease agents, allergic reactions, pain, discomfort, and stress induced losses of productivity. Because of the medical and veterinary concerns associated with *Culicoides*, the study of the genus has focused largely on the medical and veterinary aspects of these flies, resulting in gaps in our knowledge of their biology.

Less than one fifth of the immature stages of the world fauna of *Culicoides* are described (Borkent 2012b). The lack of knowledge about the taxonomy of the immature stages directly correlates with a lack of knowledge of their ecology (*e.g.,* breeding habitats, larval diet). We also know little about the evolutionary relationships of the genus. No synapomorphy of the genus has been reported. Many of the subgeneric classifications are based on studies of local faunas for which researchers assigned species to subgenera and species groups based on the similarity of species to one another (Borkent 2012b). These gaps in our knowledge of the biology of *Culicoides* are a

hindrance to the study of the genus and ecological studies. Descriptions of the immature stages will allow for larvae to be identified in ecological studies, provide phylogenetic characters, and provide data for vector surveillance and management programs. A classification system based on cladistic analysis will help stabilize the taxonomy, enhancing other ecological studies (i.e., *Culicoides* good be identified to species rather than family level) and the study of the genus.

Objectives

The objectives of my study are directed towards the larval ecology and phylogenetics of the genus. The objectives were to assess 1) the ecological variables that influence the presence-absence of larval *Culicoides* in aquatic and semiaquatic habitats of South Carolina, 2) the monophyly of the genus through cladistic analysis and broad outgroup comparison, and 3) the phylogenetic relationships among the Nearctic subgenera and species groups of *Culicoides.*

Literature review

Taxonomy

The genus *Culicoides* is composed of over 1400 species worldwide (Borkent 2012a), with 150 species found in the Nearctic region (Borkent and Grogan 2009). Taxonomic studies of the genus have been driven largely by medical and veterinary concerns and the need to identify females. As result, many of the identification keys are written for females, even though males provide more diagnostic characters to distinguish species. The lack of comprehensive identification keys for all biogeographic regions and many species still in need of description further complicate the taxonomy of the genus

(Borkent and Grogan 2009). To identify *Culicoides* specimens, one usually has to consult multiple regional keys, subgeneric or species group keys, and recent catalogues to rule out the possibility of a new species not being included in outdated keys. For adults in the Nearctic biogeographic region, identification keys at the subgenus level include the following: *Amossovia* (Wirth and Blanton 1967; as *C. guttipennis* group), *Culicoides* (Wirth and Blanton 1969; as *C. pulicaris* group), *Drymodesmyia* (Wirth and Hubert 1960; as *C. copiosus* group), *Hoffmania* (Fox 1948), and *Selfia* (Atchley 1970); and species group keys include: *C. chaetophthalmus* group (Wirth et al. 1985a), *C. debilipalpis* group (Vitale et al. 1981), *C. haematopotus* group (Atchley and Wirth 1979), *C. mohave* group (Wirth and Moraes 1979), *C. palmerae* group (Wirth and Rowley 1971), *C. piliferus* group (Wirth and Hubert 1962), *C. pusillus* group (Wirth and Mullens 1992), and *C. stonei* group (Jones and Wirth 1978). These keys require the user to know the subgenus or species group to which a specimen belongs. Identification to subgeneric level is a difficult task even for ceratopogonid experts. Downes and Wirth (1981) provided a partial key to subgenera of *Culicoides,* but this included only 11/13 and 0/7 recognized subgenera and species groups in North America north of Mexico, respectively (Borkent and Grogan 2009). Many regional keys are available, including keys for species of Alaska (Wirth 1951), New York state (Jamnback 1965), New Mexico (Atchley 1967), Missouri (Childers and Wingo 1968), Virginia (Battle and Turner 1971), and Florida (Blanton and Wirth 1979). The age of these works are a limitation, as new species distributions and new species have been documented. Aside from these keys, another

useful tool for identifying *Culicoides* is the photographic wing atlas of Wirth et al. (1985b).

Identifying immatures to the genus level is problematic. Ceratopogonidae is the only family of nematocerous Diptera without a key to genera for the larvae or pupae (Borkent and Grogan 2009), but a key to genera for the pupae is in preparation (A. Borkent personal communication). Currently there is no comprehensive key to species for immature *Culicoides.* Studies of the immature stages can reveal cryptic species, provide phylogenetic characters, enhance ecological studies, and provide data for vector management and surveillance.

Pupae can easily be associated with adults by placing a single pupa on a piece of moist filter paper and letting the adult emerge. Like the adults, there is no large-scale study of pupal *Culicoides* that includes broad geographic areas and species. Jamnback (1965) and Blanton and Wirth (1979) provided keys for pupae from New York state and Florida, respectively. Jones (1961) provided the broadest geographic study of pupal *Culicoides* in the Nearctic region, but only 13 of the 150 species (8.7%) known from the region were included. Lamberson et al. (1992) provided a key to tree-hole inhabiting *Culicoides* (14 species) in the eastern United States. Even though the pupal stage is the best known of the immature stages, much is still unknown for the taxonomy of and biology of many species of *Culicoides*.

There are a few keys to larval *Culicoides*. Jamnback (1965) provided a key to 17 species from New York. Blanton and Wirth (1979) provided a key to 12 species from Florida. The most comprehensive work in the Nearctic region was provided by Murphree

and Mullen (1991). Their study included 49 species, roughly one third of the fauna in North America north of Mexico. Much work still needs to be done on the larval taxonomy. Improvements to our understanding of larval taxonomy will facilitate larval ecology studies and provide potential phylogenetic characters.

Phylogeny

The Ceratopogonidae belong to the infraorder Culicomorpha. Within this infraorder, there are two superfamilies: the Culicoidea composed of the Culicidae, Chaoboridae, Corethrellidae, and Dixidae, and the Chironomoidea, composed of the Chironomidae, Ceratopogonidae, Simuliidae, and Thaumaleidae (Wood and Borkent 1989). The monophyly of the infraorder and superfamilies are well supported by morphological (Wood and Borkent 1989, Oosterbroek and Courtney 1995) and molecular evidence (Pawlowski et al. 1996), but the relationships of the families of Chironomoidea have been contentious. Within the infraorder, Ceratopogonidae have been placed as the sister group to Chironomidae (Wood and Borkent 1989, Beckenbach and Borkent 2003), Simuliidae (Hennig 1973), Chironomidae+Simuliidae (Saether 2000), and Simuliidae+Thaumaleidae (Pawlowski 1996).

The family Ceratopogonidae is divided into four subfamilies. The monophyly of these subfamilies is well resolved, supported by morphological evidence from extant species and an extensive fossil transition series (Borkent 1995, Borkent 2000) and molecular evidence (Beckenbach and Borkent 2003). The genus *Culicoides* belongs to the subfamily Ceratopogoninae and the tribe Culicoidini. The tribe Culicoidini is composed of the extant genera *Culicoides*, *Paradasyhelea*, and *Washingtonhelea*

(Borkent 2005). The relationships among these three genera are unresolved, but the tribe is sister to the remaining Ceratopogoninae (Borkent 2005).

The phylogenetic relationships within the genus *Culicoides* are in great need of revision. Numerous authors have divided the genus into subgenera and species groups (Root and Hoffman 1937; Edwards et al. 1939; Fox 1948, 1955; Vargas 1953; Khalaf 1954). In their catalog of world biting midges, Borkent and Wirth (1997) listed 35 subgenera of *Culicoides*. In the Nearctic region, 13 subgenera and 7 species groups are known (Borkent and Grogan 2009). The current classification is based largely on overall similarities among species, as opposed to using uniquely shared characters. Khalaf (1954) was the first to examine the relationships among the subgenera, but these relationships were based on phenetic similarities rather than cladistic synapomorphies. Recent outbreaks of bluetongue virus in Europe (Melhorn et al. 2007) have renewed interest in the phylogenetic relationships among *Culicoides*, especially the subgenera *Culicoides* and *Avaritia*, the subgenera containing the vectors of bluetongue virus in Europe. These recent studies have focused on molecular characters to infer phylogenetic relationships, including mitochondrial DNA (Dallas et al. 2003; Nolan et al. 2007; Pages et al. 2009) and nuclear ribosomal DNA (Gomulski et al. 2005; Perrin et al. 2006; Matsumoto et al. 2009; Schwenkenbecher et al. 2009). The number of taxa in many of these studies is low (usually 1-2 subgenera), further studies that include a greater breadth of subgenera and species groups are needed to understand the phylogeny of the genus.

Structure and Function

Adult

Head.—(Fig. 1.1). The compound eyes are large and form the majority of the head. They are contiguous to slightly separated and the degree of separation can be a diagnostic character at the species level. Between the eyes, separating the frons from the vertex is the arched supraorbital suture, which is absent in some species (e.g., subgenus *Avaritia*) (Battle and Turner 1971). Ventral to the superior transverse suture is the median bristle or interocular seta. The frons separates the antennae and is less prominent in males than in females. Ocelli are lacking in all families of the suborder Culicomorpha (Wood and Borkent 1989), but raised areas above the antennae and adjacent to the frons have been hypothesized to be possible ocelli (Jobling 1928; Blanton and Wirth 1979; Downes and Wirth 1981) in Ceratopogonidae. The function of these structures is not known, but they are likely not ocelli as they are fleshy and lack a lens. Ventral of the frons and antennae is the clypeus, which articulates with the mouthparts that form the proboscis. More detailed accounts of the head and mouthparts were provided by Carter et al. (1920), Jobling (1928), and Gad (1951).

The antennae of *Culicoides* hold a wealth of taxonomic, phylogenetic, and ecologic information. The antennae are composed of three segments: the basal, ring-like scape, a large pedicel, and the flagellum that is secondarily segmented into 13 flagellomeres. In the older literature, the antennae are often referred to as having 15 segments, treating the secondary segments of the flagellum as true segments. The antennae are sexually dimorphic. The male antennae each have a large setal plume

composed of whorls of elongate verticils on flagellomeres 1-10. In some species, such as *C. leechi* Wirth, 1977, and *C. utahensisi* Fox, 1946, the antennal plume is absent and the antennae resemble those of the female (Wirth and Rowley 1971; Wirth 1977). Five types of sensilla are found on the antennae of *Culicoides*: sensilla chaetica, sensilla trichodea, sensilla basiconica, sensilla ampullaceal, and sensilla coeloconica and are found in both sexes (Chu-Wang et al. 1975; Wirth and Navai 1978; Felippe-Bauer et al. 1989; Blackwell et al. 1992). The sensilla chaetica function as mechanoreceptors or mechano- and chemoreceptors and form the verticils on the antennae (Wirth and Navai 1978). The sensilla trichodea and basiconica are likely chemoreceptors and have been used little in the classification and taxonomy of *Culicoides,* though in some species their presence-absence and arrangement can be informative (Wirth and Navai 1978). The sensilla ampullaceae are somewhat difficult to see with light microscopy and their function is not known. The flagellomeres bearing sensilla coeloconica are of taxonomic significance and have been used extensively in developing the classification of *Culicoides.* These sensilla also are indicators of host association. Species that bear sensilla coeloconica on 8-13 flagellomeres are generally ornithophilic, and those that bear sensilla on 4-6 flagellomeres are generally mammalophilic (Jamnback 1965; Chu-Wang et al. 1975; Braverman and Hulley 1979; Felippe-Bauer et al. 1989; Blackwell et al. 1992). In mosquitoes, these sensilla coeloconica function as thermoreceptors responding to changes in temperature (Davis and Sokolove 1975). The same function is hypothesized for *Culicoides* (Wirth and Navai 1978), but these sensilla coeloconica have been shown to respond to carbon dioxide and humidity (Blackwell et al. 1992). If these do function as

thermoreceptors, one might expect sensilla coeloconica presence to differ among species that feed on poikilothermic and homeothermic hosts, but no such pattern has been observed (Borkent 1995). Further studies are necessary to assess the role sensilla coeloconica in host location.

Located on either side of the proboscis are the maxillary palps. These appendages, like the antennae, provide valuable taxonomic and ecological information. In *Culicoides*, the palps are five-segmented. The third segment bears a number of sensilla basiconica (referred to as capitate sensilla in some of the literature, *e.g.,* Borkent 1995) located in a pit, irregular patch, or spread over the entire surface of the segment. The palps are sexually dimorphic, with females having larger, more developed third segments. The ratio of the length to width of the third segment and the shape and depth of the sensory pit or sensory area are taxonomically informative. The sensilla basiconica are sensitive to changes in carbon-dioxide concentration (Grant and Kline 2003), an important cue in host location. The number of sensilla is related to host size and can be a predictor of host associations (Rowley and Cornford 1972). Those species that feed on small hosts tend to have more sensilla (>29) than those that feed on larger hosts (<25) (Rowley and Cornford 1972; Chu-Wang et al. 1975; Braverman and Hulley 1979). The adaptive advantage of having more sensilla is presumably to detect lower outputs of carbon dioxide from small hosts. In contrast, species that feed on large hosts do not need as many receptors to detect the greater output of carbon dioxide. The shape of the third palpal segment also can be an indicator of host association (Borkent 1995). The third segment of mammal-feeding species tends to be elongate and slender, those of bird-feeding species tend to be shorter

and wider, and those of non-biting species are short and squat (Borkent 1995). The shape of the third palpal segment is not as strong an indicator as the number of sensilla basiconica and no causative explanation for this correlation has been invoked other than it might simply be the result of the number of sensilla (more sensilla need more space) (Borkent 1995).

The mouthparts of adults are elongated into a proboscis formed from anterior to posterior by the labrum, mandibles, hypopharynx, laciniae of the maxilla, and labium. The following describes the form and function of the mouthparts in the blood-feeding species *C. sanguisuga* (Coquillet), 1901 (Sutcliffe and Deepan 1988). The labrum arches anteriorly and is composed of a central cuticular strip and two lateral flaps. The distal tip of the labrum bears a series of lateral teeth and a pair of apical tricuspid teeth. Posterior to the labrum is a pair of distally serrate mandibles. The mandibles overlap each other and interlock by means of a posterior cuticular projection on the superior mandible (left mandible in *C. sanguisuga*) that fits in with a depression on the inferior mandible. Besides serving as the cutting apparatus for skin penetration, the mandibles serve as the floor of the food channel, formed with the labrum, and the ceiling of the salivary channel, formed with the hypopharynx. Like the labrum, the hypopharynx bears a series of teeth along its distal tip. Posterior to the hypopharynx are the laciniae of the maxillae. The laciniae wrap around the edges of the hypopharynx and mandibles medially and are armed distally with retrorse teeth. The posterior of the proboscis is formed by the labium which wraps around the other mouthparts, especially at the distal tip.

After studying the structure of the mouthparts, Sutcliffe and Deepan (1998) proposed the following mechanism by which *Culicoides* penetrate vertebrate epithelium. After locating a host and suitable biting site, the proboscis is engaged with the skin. The labrum flexes anteriorly and the hypopharynx flexes posteriorly, stretching the epithelium taut. The armature at the distal tips of these mouthparts help grip and stretch the skin. The mandibles are then retracted, pulling the serrated teeth against the skin. Little abduction and adduction likely occurs because of the interlocking mechanism of the mandibles and the interaction with the other mouthparts. Protraction of the mandibles occurs by cuticular elasticity. The lack of muscular protraction of the mandibles is logical because mandibles of Ceratopogonidae bear teeth on only the outer surface; muscular protraction would not result in additional cutting and could hinder protraction. After the initial penetration, the laciniae are protracted into the wound and the retrorse teeth used to grip the tissue as the laciniae are retracted, pulling the mouthparts deeper into the wound. The process is then repeated until a pool of blood is formed. During blood feeding, blood is sucked up the food channel formed by the labrum and mandibles by the powerful cibarial pump. Simultaneously, saliva that contains anticoagulants and vasodilators is delivered by the salivary channel formed from the mandibles and hypopharynx. At the completion of blood feeding, the mouthparts are disengaged and the midge leaves the host.

The structure of the mouthparts can provide ecological information. Non-biting species tend to lack labral, mandibular, hypopharyngeal, and lacinial teeth (Borkent 1995). The distal tip of the labrum is also fleshy and not well sclerotized (Jamnback 1965). The mandibular teeth can indicate the type of host upon which a particular species

of *Culicoides* feeds. A few species feed on invertebrate hosts (Laird 1946, Wirth and Hubert 1989). These species have several large, coarse teeth, while those that feed on vertebrates have more small and fine teeth (Borkent 1995). In flies that specialize on amphibians, the mandibles are finely serrate, but the laciniae lack teeth (Borkent 1995).

Thorax.—The thorax consists of the sclerites and appendages of the pro-, meso-, and metathorax; the legs, wings, and halters. Within *Culicoides,* characters from the thoracic sclerites have not been widely used for taxonomic purposes. The prominent prescutal pits have been used as a diagnostic character for the genus (Downes and Wirth 1981), though this character can be somewhat difficult to distinguish and is found in other genera. The color patterns of the scutum, scutellum, and post scutellum have been used in species diagnosis, as well as the pollinosity of the scutum (Blanton and Wirth 1979). There are likely other characters of diagnostic and phylogenetic value in need of description on the thorax.

The homology of the wing veins has been a subject of debate in the Ceratopogonidae. The current accepted nomenclature is that of Szadziewski (1996) and was summarized and compared to previous works in Spinelli and Borkent (2004) (Fig. 1.3). By comparing fossil ceratopogonids to other extinct and extant Culicomorpha, Szadziewski (1996) was able to elucidate the homologies of ceratopogonid wing veins. The costa forms the anterior margin of the wing. The subcosta is reduced to absent. Vein R_1 and the radial sector are compact, joining with the costa at approximately the midpoint of the wing (Fig. 1.3A). In most species of *Culicoides,* two well-developed radial cells occur (Fig. 1.3.B). Vein R_1 is the first vein to join the costa, forming the proximal

boundary of the first radial cell, R_2 is a transverse vein connecting with R_1 and divides the two radial cells, and R_3 forms the distal boundary of the second radial cell (Fig. 1.3A). Veins R_4 and R_5 have been lost in *Culicoides*, but can be observed in other extant and extinct Ceratopogonidae and referred to by some sources as the intercalary vein or false vein (Szadziewski 1996). The medial vein is well developed and forks into two branches distal to the r-m crossvein. The anterior cubitus vein is well developed and has two branches. No medio-cubitus crossvein is present. The posterior cubitus and anal veins are weakly developed and do not reach the wing margin.

The light and dark patterns of the wings are characters of significant taxonomic value in *Culicoides*. These patterns are the result of the length and pigmentation of the macrotrichia on the wing surface (Fig. 1.4) (Blanton and Wirth 1979). These patterns provide some of the basis for the current subgeneric classification system of *Culicoides*. However, the character states can be difficult to determine and are based on phenetic groupings resulting in many of the subgenera likely being polyphyletic or paraphyletic.

A leg consists of six segments: the coxa, trochanter, femur, tibia, basitarsus, and tarsus, which is secondarily segmented into four tarsomeres with the fourth bearing a pair of claws. In most of the ceratopogonid literature, these last two segments are treated as five-segmented tarsi. The current evidence indicates that the basitarsus is a true segment and segmentation observed in the tarsus is secondary segmentation (Kukalová-Peck 1992). The legs of *Culicoides* lack many of the modifications observed in other genera of Ceratopogonidae (e.g., enlarged empodium, femoral armature, tarsal batonnets). Each fore-tibia and hind tibia bear grooming structures (Linley and Cheng 1974). Each fore-

tibia bears a row of slender spines and an articulated spur distally, and each hind tibia bears two rows of spines, the proximal row slender and more numerous and the second row stout and less numerous, as well as a spur distally (Linley and Cheng 1972). The second row of spines on the hind tibia holds some taxonomic value (Blanton and Wirth 1979). The hind basitarsus bears a brush of thickened setae that also function in grooming (Linley and Cheng 1972). The claws of the female are simple, equal, and lack any of the modifications observed in some species of black flies (e.g., teeth or lobes; Adler et al 2004). The claws of males are equal with their apical tips each bifid.

Abdomen.—The abdomen is composed of 10 segments, with segments II-VII bearing spiracles (Downes and Wirth 1981). The abdominal tergites are well developed. The sternites are not as heavily sclerotized as the tergites. The pleural region is membranous, allowing for abdominal expansion during blood feeding and oogenesis. The venter of the abdomen is covered with mechano- and chemoreceptors that function in host location and oviposition (Sollai et al. 2010). These include sensilla chaetica and trichoidea. Although Sollai et al. (2010) used the terms "chaetica" and "trichoidea" interchangeably, their descriptions of the sensilla represent two distinct groups of sensilla as presented by Wirth and Navai (1978) for the antennae.

The external features of the female terminalia include a pair of hypogynial valves originating from sternite VIII and a pair of well-developed cerci articulating with segment IX (Fig. 1.5A). The external features of the female terminalia have not been used extensively in species diagnoses. In black flies, these features can provide some taxonomic information useful for species diagnosis (Adler et al. 2004). For

morphological study, the terminalia of black flies typically are placed in glycerin and viewed from different angles, while those of ceratopogonids are slide mounted and viewed in one plane. Preparing terminalia of ceratopogonids in a different manner could possibly reveal new diagnostic characters. Unlike the external features of the terminalia, the internal features are taxonomically informative. One to three spermathecae are present depending on the species and species having two spermathecae can have a rudimentary third (Blanton and Wirth 1979). The shape of the spermatheca(e), the presence of a sclerotized neck, and length and thickness of the neck are all diagnostic. The spermathecal ducts from each spermatheca converge to form a common duct. In some species, the area of the duct at this juncture is sclerotized, forming a ring.

The male terminalia (Fig. 1.5C, D) hold many taxonomic and phylogenetic features. Tergite and sternite IX are fused and articulating with these is a pair of gonopods used for grasping the female during copulation. These appendages are two segemented consisting of a proximal gonocoxite and distal gonostylus. The gonocoxite bears two processes proximally: a dorsal root and a ventral root. The dorsal root articulates with the parameres (Wirth and Blanton 1979); the function of the ventral root is undetermined. The aedeagus consists of a sclerotized plate ventrally and a membranous area dorsally. The sclerite, referred to as the aedeagus in the literature, is composed of two anteriorly directed arms that converge into a plate distally, resulting in a U-, V-, or Y-shape sclerite. The parameres consist of two rods or one fused plate depending on the species. Parameres present as two rods exhibit various modifications at the base, middle

stem, and distal tip. Fused parameres are similar in appearance to the aedeagus. A pair of small cerci is present.

Pupa

Head.—The head of the pupa bears the sheaths of the antennae and mouthparts. Dorsal to the sheaths of the mouthparts are two sets of setae: The ventrolateral setae and the ventromedial setae (Nevill and Dyce 1994). The dorsal portion of the head bears the operculum (Fig. 1.6, 1.7A), a plate that separates from the rest of the pupal cuticle during the process of eclosion. The morphology of the operculum holds much diagnostic information. The anterior half bears a pair of tubercles known as the anteromarginal tubercles, each bearing a large, strong seta. Each tubercle bears a basal sensillum. The posterior half of the operculum is known as the disc and is armed with spinules, the density and distribution of which can be diagnostic.

Thorax.—The unsegmented thorax bears the leg and wing sheaths. It also bears the respiratory organ or respiratory horn (Fig. 1.6). Spiracular openings occur at the apical tip and along the lateral margin (Fig. 1.7B). The number, positioning, and presence of spiracular openings can be diagnostic as well as the presence of scales, spinules, and folds (Blanton and Wirth 1979, Nevill and Dyce 1994). The thorax also bears two sets of tubercles, the dorsolateral tubercles ventral to the respiratory organ and the dorsal tubercles on the middle of the dorsum (Fig. 1.6).

Abdomen.—Abdominal segments 3-7 usually bear five sets of tubercles (Fig. 1.6) (Nevill and Dyce 1994). The names of the tubercles are relatively similar in the different systems used by various authors (Fig. 1.8). On the anterodorsal side, the first set of

tubercles (generally 2) is the dorsal anterosubmarginal tubercles, usually abbreviated as *dasm*. The set of tubercles (generally 5) posterior to the *dasm* is the dorsal posteromarginal tubercles (*dpm*). Laterally, the anterior tubercles are the lateral anterosubmarginal tubercles (*lasm*, generally 1) and the posterior set is the lateral posteromarginal tubercles (*lpm*, generally 3). Ventrally, there is a single set of tubercles (generally 3) called the ventral posteromarginal tubercles (*vpm*). The method by which the tubercles are numbered varies among authors. The system modified by Jones (1961) numbers the tubercles with Arabic numerals from the ventral midline dorsally for each set. The system followed by Nevill and Dyce (1994) numbers the tubercles with lower case Roman numerals from the dorsal midline ventrally for each set. The shape of the tubercles and the presence of a seta are diagnostic to the species level.

Abdominal segment IX (or the caudal segment) does not bear any tubercles. The posterior end of the segment bears two pronounced posterolateral processes (Fig. 1.6). The presence and distribution of spinules or scales on the caudal segment are diagnostic.

Larva

Larvae of *Culicoides* are long and cylindrical. The head capsule is well sclerotized. The segments of the thorax and abdomen are relatively undifferentiated from one another with the exception of the caudal segment. Murphree and Mullen (1991) gave a detailed account of the morphology of larval *Culicoides*. Here, a brief overview of the morphology will be given.

Head.—The head capsule (Fig. 1.9A) is formed from the dorsal frontoclypeus and the lateroventral wall, all fused into one complex sclerite (Murphree and Mullen 1991).

These two sclerites meet posteriorly with the collar, considered by some authors to be a separate sclerite (Murphree and Mullen 1991). Anteriorly, these sclerites are more sclerotized forming a subgenal ring, which provide the attachment points for the maxillae and mandibles. The chaetotaxy and sensilla of the head capsule are diagnostic. Various systems for naming the head capsule setae have been developed. The most commonly used system is that adopted and modified by Lawson (1951), in which letters are assigned to the 13 pairs of setae and seven sensilla.

The preoral cavity is formed from the labrum and the hypostoma. The dorsal surface of the preoral cavity is formed by the labrum, which bears a series of sensilla (Hribar 1993). The undersurface of the labrum bears moveable appendages called the messors, which have an unknown function (Hribar 1993). The ventral portion of the preoral cavity is formed by the hypostoma, a sclerotized and pointed plate that can be smooth or toothed, the pattern of which holds diagnostic information (Murphree and Mullen 1991) (Fig. 1.9B). The feeding action of *Culicoides* suggests that the hypostoma is used as a scraper to dislodge food particles from the substrate (Hribar 1993). The mandibles are indicative of the feeding habits of *Culicoides*. They are heavily sclerotized and slightly curved, and can bear 1-2 teeth on the inner margin (Murphree and Mullen 1991). The mandibles indicate a generalist feeding style intermediate between the scooplike toothed mandibles of algal and diatom grazers like *Forcipomyia* and *Dasyhelea* and the long, curved, pointed mandibles of predators like *Bezzia* (Hribar 1993).

The pharyngeal apparatus is a sclerotized structure composed of a dorsal epipharynx and ventral hypopharynx (Murphree and Mullen 1991). The epipharynx is

formed from two arms suspending a series of combs (usually 4 in *Culicoides*) above the hypopharynx. The hypopharynx is composed of a lightly sclerotized membrane spanning between two arms. Depending on the species, the posterior portion of the membrane can be armed with a series of slender spines. These two structures are thought to function as a grinding mill for ingested food (Murphree and Mullen 1991). The epipharynx holds a wealth of diagnostic information and is one of the most important structures for identifying larvae to species (Murphree and Mullen 1991).

Thorax.—The three thoracic segments exhibit various patterns of pigmentation that can be of diagnostic value. The prothorax is subdivided into the cervix and the prothorax (Murphree and Mullen 1991). The mesothorax and metathorax are similar in morphology. The chaetotaxy of the thoracic segments is of little taxonomic value and was found to be constant across the genus (Kettle and Lawson 1952, Linley and Kettle 1964).

Abdomen.—Abdominal segment 1-7 are morphologically indistinct, bearing 13 pairs of setae, and segment 8 differs only in the number of setae (9 pairs) (Murphree and Mullen 1991). Segments 1-8 are of little taxonomic significance. Segment 9 is the most distinct of the abdominal segments (Fig. 1.9E). The number of setae is variable among species and can be diagnostic. Around the anus is a series of setae called the perianal setae or perianal bristles. These setae could aid in swimming or be mechanoreceptors (Kettle and Elson 1976). The caudal segment also bears a series of eversible anal papillae. The structures were initially hypothesized to be gills, but are now viewed as osmoregulatory organs (Lawson 1951).

Egg

The eggs of *Culicoides* are elongate and slender, slightly curved or relatively straight. The anterior end contains numerous aeropyles for gas exchange (Day et al. 1997; Cribb and Chitra 1998). The posterior end of the egg also contains aeropyles, but not to the extent of the anterior. The surface of the chorion bears tubercles, also referred to as ansulae (Becker 1961), typically arranged in longitudinal rows. The density of the ansulae ranges from sparse to dense depending on the species. The morphology of the ansulae can vary with the curvature of the egg, with the convex side having smaller ansulae than the concave (Campbell and Kettle 1975; Breidenbaugh and Mullens 1999). The function of the ansulae has not been resolved. In dubbing them ansulae (Latin *ansa* – a means of holding or gripping), Becker (1961) hypothesized that they had adhesive qualities. The mechanism of gripping was hypothesized to be by adhesive secretions. Cribb and Chitra (1998) reported five layers of the chorion. The third layer represented the ansulae (tubercle meshwork). The outermost layer was determined to be proteinaceous. Day et al. (1997) reported a thin layer of adhesive over the ansulae in *C. circumscriptus* Kieffer, 1918*, C. gejgelensis* Dzhafarov, 1964*,* and *C. imicola*, Kieffer, 1913. Campbell and Kettle (1975) hypothesized that the ansulae serve as a plastron to facilitate gas exchange when the eggs are submerged and that any adhesive qualities likely were produced by the collatereal glands.

The egg is the least studied of the life stages. Further investigations into egg morphology could provide phylogenetic or ecological data. The arrangement, shape, and size of the ansulae could be clade specific. The patterns of ansulae also could be the

results of environmental pressures. Perhaps eggs laid in particular habitats have similar patterns of ansulae.

Ecology

Studies of adult ecology are primarily focused in two areas: (1) seasonality and (2) activity and blood feeding. The majority of *Culicoides* are active around dusk (Blanton and Wirth 1979), though some species show bimodal peals of activity, one around dawn and the other around dusk (Kline and Roberts 1982). Many species experience a population peak in the spring months in temperate regions, with some species occurring throughout the summer (Blanton and Wirth 1979). Some species have peak populations in spring followed by a secondary peak in the fall (Kline and Axtell 1979).

Biting midges use a series of cues to locate hosts. One of the most important cues is carbon dioxide. As vertebrates exhale, carbon dioxide is released and stimulates female midges to fly upwind towards the source (Bhasin et al. 2000). If the concentrations exceed a specific threshold, the midge responds with erratic behavior and failure to fly upwind (Bhasin et al. 2000). Jamnback (1965) predicted that the number of antennal flagellomeres bearing sensilla coeloconica is correlated with host association. Similar observations have been made for the capitate sensilla of the third palpal segment (Borkent 1995). Mammalophilic species tend to have fewer flagellomeres bearing sensilla coeloconica and fewer capitate sensilla on the palp corresponding to the amount carbon dioxide produced by the host (Jamnback 1965, Borkent 1995).

Known host associations of *Culicoides* are sparse. Many of the known host associations are based on collections from domestic animals (Hair and Turner 1968, Humphreys and Turner 1973, Tanner and Turner 1974, Koch and Axtell 1979, Schmidtmann et al. 1980). Besides observations and collections from baited animals, tests of blood-fed females using the precipitin test (Nishijima and Ono 1964) and ELISA test (Blackwell et al. 1994, Blackwell et al. 1995) have been used to identify hosts. These methods provide the host to the order or family level, but seldom to the species level. DNA technology is one solution to this problem. PCR analysis of blood meals has been used to identify hosts in mosquitoes and blackflies and recently has been applied to identifying hosts in biting midges (Bartsch et al. 2009, Garros et al. 2011, Lassen et al. 2011, Ninio et al. 2011). One issue with identifying blood meals from engorged females is finding engorged females. Blood-fed females were collected at greater frequency at 10 m above ground as compared to ground level, even after feeding on vertebrate hosts (D.A. Swanson unpublished data). Setting traps or collecting at appropriate locations will improve collection of engorged females and help identify hosts.

Adult midges generally do not disperse far from larval breeding habitats, thus distribution is largely determined by the larval habitat. The collection of adults by various monitoring methods can provide reasonable approximation of species distributions. Climatic models predicting the distribution of bluetongue vectors were able to predict the presence/absence of five *Culicoides* species for 74-87% of the sites sampled across Sicily, Italy (Purse et al. 2004). Input of additional data into models, such as land cover and livestock numbers also can enhance predictive models (Liberato et al. 2010). Being

able to predict where vector species, such as *Culicoides,* will occur allows vector surveillance and management programs to efficiently target populations of biting midges more efficiently and effectively.

The larvae of *Culicoides* occur in a variety of aquatic to semiaquatic habitats. The ecology of the larvae is best known for salt marsh species, important vector species, and tree-hole species. The larvae are generalists feeding on diatoms, algae, fungi, rotifers, oligochaetes, and other arthropods (Hribar 1993, Hribar and Mullen 1991, Aussel and Linley 1994). Populations of *Culicoides* can range from sparse to quite dense. Larval densities of *Culicoides belkini* Grogan and Wirth, 1979, collected from the Society Islands reach as high as 25,500 larvae/ m^2 (Lardeux and Ottenwaelder 1997). Besides the particular types of habitat (e.g., tree hole, salt marsh, pond), there are other environmental factors that limit the larval habitat. Plant cover is associated with the distribution of the salt marsh species, with certain species occurring in greater abundance with certain plants (Kline and Axtell 1977, Kline and Roberts 1982, Kline 1986, Kline and Wood 1988). This association with plant types might reflect differences in the amount of time the soil is inundated or in soil characteristics. Soil composition and chemistry are correlated with the distribution of the sister species *C. variipennis* (Coquillett), 1901, and *C. sonorensis* Wirth and Jones, 1957. In the Great Plains, *C. variipennis* is distributed east of the Missouri River in glaciated soils and *C. sonorensis* is distributed west of the Missouri River in non-glaciated soils (Schmidtmann et al. 2011). Other factors likely limit the distributions as *C. variipennis* also occurs in the southeastern US, where the soils were never glaciated. Higher organic loading (high phosphate, percent organic matter, and

nitrate) are good indicators for the larval habitat of *C. variipennis, C. sonorensis,* and *C. nubeculosus* (Meigen), 1830 (Schmidtmann et al. 2000, Uslu and Dik 2010). High concentrations of salt ions are correlated with the distribution of members of the *C. variipennis* complex (Schmidtmann et al. 2000).

Medical and Veterinary Importance

Members of the genus *Culicoides* are implicated as vectors for 66 viruses, 15 protozoans, and 26 filarial nematodes, as well as causing allergic reactions in hosts (Borkent 2004). Species of *Culicoides* have been implicated in the transmission of viruses in the families Bunyaviridae, Reoviridae, and Rhabdoviridae (Mullen 2009). No known bacterial pathogens are known to be transmitted by *Culicoides*.

In North America, no significant human pathogens are known from *Culicoides,* but potential vectors do occur in this region. *Culicoides paraensis* (Goeldi), 1905, ranges from Pennsylvania and Wisconsin to Argentina (Borkent and Grogan 2009). In South America, this species transmits Oropouche virus, the causative agent of a nonfatal disease characterized by fever and sever joint pain. Three vectors of *Mansonella ozzardi* (Manson), 1897, a relatively benign filarial nematode in South America and the Caribbean, occur in the United States (Mullen 2009, Borkent and Grogan 2009). *Culicoides furens* and *C. barbosai* Wirth and Blanton, 1956, are common along the eastern coast of the United States and *C. paraensis* is common throughout the eastern United States. West Nile Virus was isolated from species of *C. stellifer* (Coquillet), 1901, but transmission was not demonstrated (Sabio et al. 2006).

Culicoides are more significant veterinary pests than medical pests. The major viruses transmitted by no-see-ums are in the family Reoviridae and include African horse sickness virus, bluetongue virus, and epizootic hemorrhagic disease virus. In populations of equines with low resistance, African horse sickness can have mortality levels higher than 90% (Mellor et al. 2000). Bluetongue virus is a disease of domestic and wild ruminants. In wild ruminants and cattle, clinical symptoms are seldom exhibited when infected with bluetongue virus, but these hosts can serve as reservoirs for the virus (Mellor et al. 2000). In sheep, infection with bluetongue virus can cause mortality levels of over 75% (Mullen 2009). More important than the mortality to animals are the movement restrictions of animals in infected areas. Trade restrictions due to quarantines are estimated to cost US producers $125 million annually (Bram et al. 2002). Epizootic hemorrhagic disease is similar to bluetongue but affects primarily wild ruminants (e.g., white-tailed deer), but outbreaks do occasionally occur in cattle (Mullen 2009). Other viruses associated with *Culicoides* are Palyam viruses, Equine encephalosis virus, Bovine ephemeral fever virus, and Akabane virus (Mellor et al. 2000).

Biting midges are known vectors of blood protozoans of the genera *Haemoproteus, Hepatocystis,* and *Leucocytozoon* (Mullen 2009). Most of these parasites are benign, but some can cause acute disease in hosts. *Haemoproteus meleagridis* Levine, 1961, can cause weight loss, anemia, and organ damage in domestic turkeys (Mullen 2009). *Leucocytozoon caulleryi* Mathis and Léger, 1909, causes serious disease in poultry in southeast Asia (Mullen 2009). Some evidence indicates that species of *Culicoides* could serve as vectors of avian trypanosomes (Mullen 2009).

Studying *Culicoides*

Adults are the life stage most easily collected. Light traps baited with carbon dioxide, such as Centers for Disease Control and Prevention (CDC) miniature light traps, are a good way to collect females. Different wavelengths of light can increase the number of specimens of *Culicoides* collected and reduce collection of non-target insects (Bishop et al. 2006, Nelder et al. 2010). Traps that incorporate carbon dioxide, heat, water vapor, and other attractants also work well (Lloyd et al. 2008). Animal-baited traps provide another means of collecting adult females and provide host-association data (Bennet 1960, Hair and Turner 1968, Koch and Axtell 1979, Schmidtmann et al. 1980, Zimmerman and Turner 1983, Raich et al. 1997), but are less convenient than light traps. Male *Culicoides* are generally not attracted to carbon dioxide baited traps. Malaise traps, vehicle mounted traps, and aerial or sweep netting are methods of collecting males if performed in a location where males are present. Trap placement can have an effect on the abundance and richness of species collected. For example, placement of traps higher in forest canopies collect higher numbers of certain species than ground-level traps (Snow et al. 1958, Tanner and Turner 1974, Henry and Adkins 1975, Swanson and Adler 2010, Swanson et al. 2012). Other factors, such as host number (Garcia-Saenz et al. 2011) or amount of attractant (Bahsin et al. 2000), can influence collection numbers and species richness by increasing attractiveness or repelling midges.

Kline et al. (1975) and Hribar (1990) provide reviews and comparisons of various methods of collecting immature biting midges. These methods can be broken into three basic classes: Sieving, floating, and extracting. Sieving involves passing substrate

samples through a series of sieves and collecting immatures from the filtrate of the sieves. This method can be combined with other methods to remove larger substrate particles. Flotation methods involve placing substrate samples in an aqueous solution that causes the immatures to float to the surface where they can be collected. Various solutes used to float immatures include salts, sugars, and carbon dioxide. Extraction methods take advantage of the behavior of immatures to concentrate them in a location where they can be easily collected. Examples of extraction methods include Berlese funnel extraction, light extraction, sand extraction, and agar extraction.

The most common method to preserve *Culicoides* is by slide mounting. This requires clearing the specimen then mounting it in some medium such as Canada balsam, euparal, or some other fixative. Various methods are available for slide mounting biting midges. Wirth and Marston (1968) provided a method of treating midges in phenol and mounting them in a one-to-one solution of Canada balsam. This method works well for a large number of specimens, but it fails to remove internal tissue, making internal structures difficult to see. Borkent and Spinelli (2007) provided a detailed method of slide mounting midges by clearing with potassium hydroxide (KOH) and mounting in Canada balsam thinned with xylene. This method eliminates internal tissue, but the KOH can continue to clear the exoskeleton if the base is not completely neutralized. This method also is more time intensive than others. Swanson and Grogan (2011) used a method of clearing the specimens in warm lactic acid, transitioning to clove oil, and mounting in a mixture of clove oil and Canada balsam. The benefit of this method is that it does not require the use of harsh chemicals such as phenol or xylene. Care should be taken when

clearing specimens with lactic acid. If the solution is heated too much, structures like the antennae and palps can rupture. Adult specimens should be dissected into four parts, head, thorax, wing, and abdomen. For studies with many specimens to identify, these parts can be mounted under a single coverslip. For more detailed morphological studies, each body part should be mounted under its own coverslip. The legs also can be treated in this manner. The head should be mounted anterior side up and the abdomen ventral side up. Larvae and pupae should be mounted dorsal side up. For the pupae, one respiratory horn and the operculum should be mounted under a separate coverslip.

One issue with slide mounting specimens is that they are fixed in one plane, losing the three-dimensional structure. In a study of the genus *Brachypogon*, Swanson and Grogan (2011) found that two closely related species could be distinguished from each other based in part on lateral views of the parameres and aedeagus. Multiple views of the terminalia are used with black flies to diagnose species (Adler et al. 2004). Further investigations into the three-dimensional structure of various body regions of *Culicoides* could provide more taxonomic and phylogenetic information. This can be done by examining specimens and body parts in glycerin or using modern technology, like confocal laser scanning microscopy (Klaus et al. 2003), to assess the three-dimensional structure.

A series of measurements and ratios are useful in the study of Ceratopogonidae. In adults, these include ratios of structures on the head, wings, and legs (Blanton and Wirth 1979). The antennal ratio (AR) is the length of flagellomeres 9-13 divided by the length of flagellomeres 1-8. The proboscis to head ratio (P/H) is the length of the

proboscis from the torma to the tip of the labrum divided by the distance from the torma to the median bristle base. The palpal ratio (PR) is the length of palpal segment III divided by the width of the same segment at its widest point. The wing length is measured from the basal arculus to the tip of the wing. The length of the costa also starts from the basal arculus and ends at the tip of the costa. The costa ratio (CR) is the wing length divided by the costa length. The tarsal ratio (TR) is not commonly used with *Culicoides*, but it can have potential value. It is define as the length of the basitarsus of the hind leg divided by the length of the first tarsomere. Measurements of the larval head capsule also have taxonomic significance and these were summarized by Murphree and Mullen (1991). The head length is measured from the tip of the labrum to posterior of the postoccipital ridge. The head width is measured at the widest point of the head. The subgenal width is measured at the posterior margin of the subgenal ring. The head ratio is the head length divided by the width and the head-width ratio is the head width divided by the subgenal width.

References

Adler, P.H., D.C. Currie, and D.M. Wood. 2004. The black flies (Simuliidae) of North America. Cornell University Press, Ithaca, New York, USA, 941 pp.

Atchley, W.R. 1967. The *Culicoides* of New Mexico (Diptera: Ceratopogonidae). *University of Kansas Science Bulletin* 46: 937-1022.

Atchley, W.R. 1970. A biosystematic study of the subgenus *Selfia* of *Culicoides* (Diptera: Ceratopogonidae). *University of Kansas Science Bulletin* 49: 181-336.

Atchley, W.R. and W.W. Wirth. 1979. A review of the *Culicoides haematopotus* group in North America (Diptera: Ceratopogonidae). *Journal of the Kansas Entomological Society* 52: 524-545.

Aussel, J.-P. and J.R. Linley. 1994. Natural food and feeding behavior of *Culicoides furens* larvae (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 21: 99-104.

Bartsch, S., B. Bauer, A. Wiemann, P.-H. Clausen, and S. Steuber. 2009. Feeding patterns of biting midges of the *Culicoides obsoletus* and *Culicoides pulicaris* groups on selected farms in Brandenburg, Germany. *Parasitology Research* 105: 373-380.

Battle, F.V. and E.C. Turner, Jr. 1971. The insects of Virginia: No. 3. A systematic review of the genus *Culicoides* (Diptera: Ceratopogonidae) of Virginia with a geographic catalog of the species occurring in the eastern United States north of Florida. Virginia Polytechnic Institute and State University, Research Division Bulletin 44. 129 pp.

Beckenbach, A.T. and A. Borkent. 2003. Molecular analysis of the biting midges (Diptera: Ceratopogonidae), based on mitochondrial cytochrome oxidase subunit 2. *Molecular Phylogenetics and Evolution* 27: 21-35.

Becker, P. 1961. Observations on the life cycle and immature stages of *Culicoides circumscriptus* Kieff. (Diptera, Ceratopogonidae). *Proceedings of the Royal Society of Edinburgh* 67: 363-388.

Bennet, G.F. 1960. On some ornithophilic blood-sucking Diptera in Algonquin Park, Ontario, Canada. *Canadian Journal of Zoology* 38: 377-389.

Bhasin, A., A.J. Mordue (Luntz), and W. Mordue. 2000. Responses of the biting midge *Culicoides impunctatus* to acetone, CO2, and 1-octen-3-ol in a wind tunnel. *Medical and Veterinary Entomology* 14: 300-307.

Bishop, A.L. G.A. Bellis, H.J. MacKenzie, L.J. Spohr, R.J. Worrall, A.M. Harris, and L. Melville. 2006. Light trapping of biting midges *Culicoides* spp. (Diptera: Ceratopogonidae) with green light-emitting diodes. *Australian Journal of Entomology* 45: 202-205.

Blanton, F.S. and W.W. Wirth. 1979. The sand flies (*Culicoides*) of Florida (Diptera: Ceratopogonidae). Arthropods of Florida and Neighboring Land Areas. Volume 10. Florida Department of Consumer Services, Gainesville, Florida, USA. 204 pp.

Blackwell, A., M. Brown, and W. Mordue. 1995. The use of an enhanced ELISA method for the identification of *Culicoides* bloodmeals in host-preference studies. *Medical and Veterinary Entomology* 9: 214-218.

Blackwell, A., A.J. Mordue (Luntz), W. Mordue. 1992. Morphology of the antennae of two species of biting midge: *Culicoides impunctatus* (Goetghebuer) and *Culicoides nubeculosus* (Meigen) (Diptera, Ceratopogonidae). *Journal of Morphology* 213: 85-103.

Blackwell, A., A.J. Mordue (Luntz), and W. Mordue. 1994. Identification of bloodmeals of the Scottish biting midge, *Culicoides impunctatus,* by indirect enzyme-linked immunosorbent assay (ELISA). *Medical and Veterinary Entomology* 8: 20-24.

Borkent, A. 1991. A new key to some genera of Ceratopogonini in the Holarctic (Diptera: Ceratopogonidae). Entomologica Scandinavica 22: 433–436.

Borkent, A. 1995. Biting midges in the Cretaceous amber of North America (Diptera: Ceratopogonidae). Backhuys Publishers, Leiden. 237 pp.

Borkent, A. 2004. The biting midges, the Ceratopogonidae (Diptera), pp. 113-126. In, W.C. Marquardt, W.C. Black IV, S. Higgs, J.E. Freier, A.A. James, H.H. Hagedorn, B. Kondratieff, J. Hemingway, C.G. Moore (Editors). Biology of Disease Vectors, 2nd Ed. Elsevier Academic Press, New York.785 pp.

Borkent, A. 2012a. World Species of Biting Midges (Diptera: Ceratopogonidae). Illinois Natural History Museum (http://www.inhs.illinois.edu/research/FLYTREE/CeratopogonidaeCatalog.pdf). [accessed 28 February 2012].

Borkent, A. 2012b. The subgeneric classification of *Culicoides* – thoughts and a warning. Illinois Natural History Museum (http://www.inhs.illinois.edu/research/FLYTREE/CulicoidesSubgenera.pdf). [accessed 4 June 2012].

Borkent, A. and W. L. Grogan, Jr. 2009. Catalog of the New World biting midges north of Mexico (Diptera: Ceratopogonidae). *Zootaxa* 2273: 1–48.

Borkent, A. and G.R. Spinelli. 2007. Neotropical Ceratopogonidae (Diptera: Insecta). 198 pp. In: J. Adis, J.R. Arias, G. Rueda-Delgado, and K.M. Wantzen. Aquatic Biodiversity in Latin America (ABLA). Vol. 4. Pensoft, Sofia-Moscow.

Borkent, A. and W.W. Wirth. 1997. World species of biting midges (Diptera: Ceratopogonidae). *Bulletin of the American Museum of Natural History* No. 233, 257 pp.

Bram, R.A., J.E. George, R.E. Reichard, and W.J. Tabachnick. 2002. Threat of foreign arthropod-borne pathogens to livestock in the United States. *Journal of Medical Entomology* 39: 405-416.

Braverman, Y. and P.E. Hulley. 1979. The relationship between the numbers and distribution of some antennal and palpal sense organs and host preference in some *Culicoides* (Diptera: Ceratopogonidae) from South Africa. *Journal of Medical Entomology* 15: 419-424.

Breidenbaugh, M.S. and B.A. Mullens. 1999. Two new western Nearctic *Culicoides* Latreille (Diptera: Ceratopogonidae) described from all stages. *Proceedings of the Entomological Society of Washington* 101: 149-163.

Campbell, M.M. and D.S. Kettle. 1975. Oogenesis in *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae) and the development of a plastron-like layer on the egg. *Australian Journal of Zoology* 23: 203-218.

Carter, H.F.A., A. Ingram, and J.W.S. Macfie. 1920. Observations on the Ceratopogonine midges of the Gold Coast with descriptions of new species. *Annals of Tropical Medicine and Parasitology* 14: 187-210.

Childers, C.C. and C.W. Wingo. 1968. Genus *Culicoides* (Diptera: Ceratopogonidae) in central Missouri: Species; seasonal abundance; activity. Research Bulletin 934, University of Missouri College of Agriculture. 32 pp.

Chu-Wang, I.-W. R.C. Axtell, D.L. Kline. 1975. Antennal and palpal sensilla of the sand fly *Culicoides furens* (Poey) (Diptera: Ceratopogonidae). *International Journal of Insect Morphology and Embryology* 4: 131-149.

Cribb, B.W. and E. Chitra. 1998. Ultrastructure of the eggs of *Culicoides molestus* (Diptera: Ceratopogonidae). *Journal of the American Mosquito Control Association* 14: 363-368.

Dallas, J.F., R.H. Cruickshank, Y.-M. Linton, D.V. Nolan, M. Patakakis, Y. Braverman, R Capela, I Pena, R. Meiswinkel, M.D. Ortega, M. Baylis, P.S. Mellor, and A.J. Mordue (Luntz). 2003. Phylogenetic status and matrilineal structure of the biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. *Medical and Veterinary Entomology* 17: 379-387.

Davis, E.E. and P.G. Sokolove. 1975. Temperature responses of antennal receptors of the mosquito, *Aedes aegypti*. *Journal of Comparative Physiology* 96: 223-236.

Day, J.F., D. Duzak, Y. Braverman, A. Chizov-Ginzburg, and J.R. Linley. 1997. Ultrastructure of the eggs of *Culicoides circumscriptus, Culicoides gejgelensis,* and *Culicoides imicola* (Diptera: Ceratopogonidae). *Journal of the American Mosquito Control Association* 13: 76-83.

Downes, J. A. and W. W. Wirth. 1981. Chapter 28. Ceratopogonidae, pp. 393–421. *In* J. F. McAlpine, B. V. Peterson, G. E. Shewell, H. J. Teskey, J. R. Vockeroth, and D. M. Wood, eds. Manual of Nearctic Diptera, Vol. 1. Agriculture Canada Monograph, 27. 264 pp.

Edwards, F.W., H. Oldroyd, and J. Smart. 1939. British blood-sucking flies. British Museum of Natural History, London, 156 pp., 45 pl.

Felippe-Bauer, M.L.; P.G. Bauer, and F.C.S. Filho. 1989. Scanning electron microscopy of the antennal sensilla in female *Culicoides paraensis* (Diptera: Ceratopogonidae). *Memorias do Instituto Oswaldo Cruz* 84: 463-469.

Fox, I. 1948. Hoffmania, a new subgenus in *Culicoides* (Diptera: Ceratopogonidae). *Proceedings of the Biological Society of Washington* 61: 21-28.

Fox, I. 1955. A catalogue of the bloodsucking midges of the Americas (*Culicoides, Leptoconops*, and *Lasiohelea*) with keys to the subgenera and Nearctic species, a Geographic index, and a bibliography. *Journal of Agriculture of University of Puerto Rico* 39: 214-285.

Gad, A.M. 1951. The head-capsule and mouthparts in the Ceratopogonidae (Diptera-Nematocera). *Bulletin de la Société Fouad 1er d'entomologie* 35 17-75.

Garcia-Saenz, A., P. McCarter, and M. Baylis. 2011. The influence of host number on the attraction of biting midges, *Culicoides* spp., to light traps. *Medical and Veterinary Entomology* 25: 113-115.

Garros, C., L. Gardes, X. Allene, I. Rakotoarivony, E. Viennet, S. Rossi, and T. Balenghien. 2011. Adaptation of a species-specific mulitplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of Orbiviruses. *Infection, Genetics and Evolution* 11: 1103-1110.

Gomulski, L.M., R. Meiswinkel, J.-C. Delecolle, M. Goffredo, and G. Gasperi. 2005. Phylogenetic relationships of the subgenus *Avaritia* Fox, 1955 including *Culicoides obsoletus* (Diptera, Ceratopogonidae) in Italy based on internal transcribed spacer 2 ribosomal DNA sequences. *Systematic Entomology* 30: 619-631.

Grant, A.J. and D.L. Kline. 2003. Electrophysiological responses from *Culicoides* (Diptera: Ceratopogonidae) to stimulation with carbon dioxide. *Journal of Medical Entomology* 40: 284-293.

Hair, J.E. and E.C. Turner, Jr. 1968. Preliminary host preference studies on Virginia *Culicoides* (Diptera: Ceratopogonidae). *Mosquito News* 28: 103-107.

Hennig, W. 1973. Ordnung Diptera (Zweiflüger). *Handbuck der Zoologie* 4: 1-337.

Henry, L.G. and T.R. Adkins, Jr. 1975. Vertical distribution of biting midges in Coastal South Carolina. *Annals of the Entomological Society of America* 68: 321-324.

Hribar, L.J. 1990. A review of methods for recovering biting midge larvae (Diptera: Ceratopogonidae) from substrate samples. *Journal of Agricultural Entomology* 7: 71-77.

Hribar, L.J. 1993. Mouthpart morphology and feeding behavior of biting midge larvae (Diptera: Ceratopogonidae). pp. 43-58. *In* Schaefer, C.W. and R.A.B. Leschen (eds). Functional morphology of insect feeding. Thomas Say Publications in Entomology, Entomological Society of America, Lanham, Maryland

Hribar, L.J. and G.R. Mullen. 1991. Alimentary tract contents of some biting midge larvae (Diptera: Ceratopogonidae). *Journal of Entomological Science* 26: 430-435.

Humphreys, J.G. and E.C. Turner, Jr. 1973. Blood-feeding activity of female *Culicoides* (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 10: 79-83.

Jamnback, H. 1965. The *Culicoides* of New York state (Diptera: Ceratopogonidae). New York State Museum Science Service, Albany Bulletin No. 399**:** 154 pp.

Jobling, B. 1928. The structure of the head and mouth parts in *Culicoides pulicaris,* L. (Diptera Nematocera). *Bulletin of Entomological Research* 18: 211-245.

Jones, R.H. 1961. Descriptions of pupae of thirteen North American species of *Culicoides* (Diptera: Ceratopogonidae). *Annals of the Entomological Society of America* 54: 729-746.

Jones, R.H. and W.W. Wirth. 1978. A new species of western *Culicoides* of the *stonei* group (Diptera: Ceratopogonidae). *Entomological News* 89: 56-58.

Kettle, D.S. and J.W.H. Lawson. 1952. The early stages of British biting midges *Culicoides* Latreille (Diptera: Ceratopogonidae) and allied genera. *Bulletin of Entomological Research* 43: 421-467, XIX plates.

Khalaf, K.T. 1954. The speciation of the genus *Culicoides* (Diptera, Heleidae). *Annals of the Entomological Society of America* 47: 34-51.

Klaus, A. V., V. L. Kulasekera, and V. Schawaroch. 2003. Three-dimensional visualization of insect morphology using confocal laser scanning microscopy. *Journal of Microscopy* 212: 107–121.

Kline, D.L. 1986. Seasonal abundance of adult *Culicoides* spp. (Diptera: Ceratopogonidae) in a salt marsh in Florida, USA. *Journal of Medical Entomology* 23: 16-22.

Kline, D.L. and R.C. Axtell. 1976. Salt marsh *Culicoides* (Diptera: Ceratopogonidae): species, seasonal abundance and comparisons of trapping methods. *Mosquito News* 36: 1-10.

Kline, D.L. and R.C. Axtell. 1977. Distribution of *Culicoides hollensis*, *C. furens*, and *C. bermudensis* in relation to plant cover in a North Carolina salt marsh (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 13: 545-552.

Kline, D.L., J.C. Dukes, and R.C. Axtell. 1975. Salt marsh *Culicoides* (Diptera: Ceratopogonidae): Comparison of larval sampling methods. *Mosquito News* 35: 147-150.

Kline, D.L. and R.H. Roberts. 1982. Daily and seasonal abundance of *Culicoides* spp. biting midges (Diptera: Ceratopogonidae) in selected mangrove areas in Lee County, Florida. *Flordia Entomologist* 65: 126-135.

Kline, D.L. and J.R. Wood. 1988. Habitat preference of Coastal *Culicoides* spp. at Yankeetown, Florida. *Journal of the American Mosquito Control Association* 4: 456-465.

Koch, H.G. and R.C. Axtell. 1979. Attraction of *Culicoides furens* and *C. hollensis* (Diptera: Ceratopogonidae) to animal hosts in a salt marsh habitat. *Journal of Medical Entomology* 15: 494-499.

Kukalová-Peck, J. 1992. The "Uniramia" do not exist: the ground plan of the Pterygota as revealed by Permian Diaphanopterodea from Russia (Insecta: Paleodictyopteroidea). *Canadian Journal of Zoology* 70: 236-255.

Laird, M. 1946. A Ceratopogonine midge (*Culicoides anophelis* Edwards, 1922) sucking engorged blood from a mosquito (*Armigeres lacuum* Edwards, 1922) at Palmalmal, New Britain. *Transactions of the Royal Society of New Zealand* 76: 158-161.

Lamberson, C., C.D. Pappas, and L.G. Pappas. 1992. Pupal taxonomy of the tree-hole *Culicoides* (Diptera: Ceratopogonidae) in eastern North America. *Annals of the Entomological Society of America* 85: 111-120.

Lardeux, F.J.R. and T. Ottenwaelder. 1997. Density of larval *Culicoides belkini* (Diptera: Ceratopogonidae) in relation to physicochemical variables in different habitats. *Journal of Medical Entomology* 34: 387-395.

Lassen, S.B., S.A. Nielsen, H. Skovgård, and M. Kristensen. 2011. Molecular identification of bloodmeals from biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) in Denmark. *Paristology Research* 108: 823-829.

Lawson, J.W.H. 1951. The anatomy and morphology of the early stages of *Culicoides nubeculosus* Meigen (Diptera: Ceratopogonidae = Heleidae). *Transactions of the Royal Entomological Society London* 102: 511-570.

Liberato, C., F. Farina, A. Magliano, P. Rombola, F. Scholl, V. Spallucci, and P. Scaramozzino. 2010. Biotic and abiotic factors influencing distribution and abundance of *Culicoides obsoletus* group (Diptera: Ceratopogonidae) in central Italy. *Journal of Medical Entomology* 47: 313-318.

Linley, J.R. and L. Cheng. 1974. The grooming organs of *Culicoides* (Diptera: Ceratopogonidae). *Mosquito News* 34: 204-206.

Linley, J.R. and D.S. Kettle. 1964. A description of the larvae and pupae of *Culicoides furens* Poey, and *Culicoides hoffmani* Fox (Diptera: Ceratopogonidae). *Annals and Magazine of Natural History*7: 129-149.

Lloyd, A.M., D.L. Kline, J.A. Hogsette, P.E. Kaufman, and S.A. Allan. 2008. Evaluation of two commercial traps for the collection of *Culicoides* (Diptera: Ceratopogonidae). *Journal of the American Mosquito Control Association* 24: 253-262.

Mathieu, B., A. Perrin, T. Baldet, J.-C. Delecolle, E. Albina, C. Cetre-Sossah. 2007. Molecular identification of Western European species of obsoletus complex (Diptera: Ceratopogonidae) by an internal transcribed spacer-1 rDNA multiplex polymerase chain reaction assay. *Journal of Medical Entomology* 44:1019-1025.

Matsumoto, Y. T. Yanase, T. Tsuda, and H. Noda. 2009. Characterization of internal transcribed spacer (ITS1)-ITS2 region of ribosomal RNA gene from 25 species of *Culicoides* biting midges (Diptera: Ceratopogonidae) in Japan. *Journal of Medical Entomology* 46: 1099-1108.

Mellor, P.S., J. Boorman, and M. Baylis. 2000. *Culicoides* Biting Midges: Their role as arbovirus vectors. *Annual Review of Entomology* 45: 307-340.

Melville, L.F., N.T. Hunt, G. Bellis, and D. Pinch. 2004. An assessment of insecticides to minimize the transmission of arboviruses in cattle. *Arbovirus Research in Australia* 8: 249-255.

Mullen, G.R. 2009. Chapter 12. Biting midges (Ceratopogonidae), pp. 169-188. *In* G.R. Mullen and L.A. Durden, eds. Medical and Veterinary Entomology 2nd Edition. Academic Press, New York, USA. 637 pp.

Murphree, C.S. and G.R. Mullen. 1991. Comparative larval morphology of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) in North America with a key to species. *Bulletin of the Society of Vector Ecologists* 16: 269-399.

Nelder, M.P., D.A. Swanson, P.H. Adler, W.L. Grogan, Jr. 2010. Biting midges of the genus *Culicoides* in South Carolina zoos. *Journal of Insect Science* 10: 1-9.

Nevill, H. and A.L. Dyce. 1994. Afrotropical *Culicoides*: Description and comparison of the pupae of seven species of the Similis supergroup (Diptera: Ceratopogonidae). *Onderstepoort Journal of Veterinary Research* 61: 85-106.

Ninio, C., D. Augot, J.-C. Delecolle, B. Dufour, and J. Depaquit. 2011. Contribution to the knowledge of *Culicoides* (Diptera: Ceratopogonidae) host preferences in France. *Parasitology Research* 108: 657-663.

Nishijima, Y. and H. Ono. 1964. Identification of blood meals of heleid biting midges by the precipitin test (Diptera). *Sanitary Zoology* 15: 131-135.

Nolan, D.V., S. Carpenter, J. Barber, P.S. Mellor, J.F. Dallas, A.J. Mordue (Luntz), and S.B. Piertney. 2007. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Veterinary Microbiology* 124: 82-94.

Oosterbroek, P. and G. Courtney. 1995. Phylogeny of the nematocerous families of Diptera (Insecta). *Zoological Journal of the Linnean Society* 115: 267-311.

Pagès, N., F. Muñoz-Muñoz, S. Talavera, V. Sarto, C. Lorca, and J.J. Núñez. 2009. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. *Veterinary Parasitology* 165: 298-310.

Pawlowski, J., R. Szadziewski, D. Kmieciak, J. Fahrni, and G. Bittar. 1996. Phylogeny of the infraorder Culicomorpha (Diptera: Nematocera) based on 28s RNA gene sequences. *Systematic Entomology* 21: 167-178.

Perrin, A., C. Cetre-Sossah, B. Mathieu, T. Baldet, J.-C. Delecolle, and E. Albina. 2006. Phylogenetic analysis of *Culicoides* species from France based on nuclear ITS1-rDNA sequences. *Medical and Veterinary Entomology* 20: 219-228.

Purse, B.V., A.J. Tatem, S. Caracappa, D.J. Rogers, P.S. Mellor, M. Baylis, and A. Torina. 2004. Modeling the distributions of *Culicoides* bluetongue virus vectors in Sicily in relation to satellite-derived climate variables. *Medical and Veterinary Entomology* 18: 90-101.

Raich, T., M. Jacobson, F. Holbrook, R. Rabion, C. Blair, and B. Beaty. 1997. *Culicoides variipennis* (Diptera: Ceratopogonidae) host selection in Colorado. *Journal of Medical Entomology* 34: 247-249.

Root, F.M., and W.A. Hoffman. 1937. The North American species of *Culicoides*. *The American Journal of Hygiene* 25: 150-184.

Rowely, W.A. and M. Cornford. 1972. Scanning electron microscopy of the pit of the maxillary palp of selected species of *Culicoides. Canadian Journal of Zoology* 50: 1207-1210.

Sabio, I.J., A.J. Mackay, A. Roy, and L.D. Foil. 2006. Detection of West Nile Virus RNA in pools of three species of ceratopogonids collected in Louisiana. *Journal of Medical Entomology* 43: 1020-1022.

Saether, O.A. 2000. Phylogeny of Culicomorpha (Diptera). *Systematic Entomology* 25: 223-234.

Schmidtmann, E.T., R.J. Bobian, and R.P. Belden. 2000. Soil chemistries define aquatic habitats with immature populations of the *Culicoides variipennis* complex (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 37: 58-64.

Schmidtmann, E.T., M.V. Herrero, A.L. Green, D.A. Dargatz, J.M. Rodriquez, and T.E. Walton. 2011. Distribution of *Culicoides sonorensis* (Diptera: Ceratopogonide) in Nebraska, South Dakota, and North Dakota: Clarifying the epidemiology of bluetongue disease in the northern great plains region of the United States. *Journal of Medical Entomology* 48: 634-643.

Schmidtmann, E.T., C.J. Jones, and B.Gollands. 1980. Comparative host-seeking activity of *Culicoides* (Diptera: Ceratopogonidae) attracted to pastured livestock in Central New York state, USA. *Journal of Medical Entomology* 17: 221-231.

Schwenkenbecher, J.M. A.J. Mordue (Luntz), and S.B. Piertney. 2009. Phylogenetic analysis indicates that *Culicoides dewulfi* should not be considered part of the *Culicoides obsoletus* complex. *Bulletin of Entomological Research* 99: 371-375.

Snow, W.E., E. Pickard, and C.M. Jones. 1958. Observations on the activity of *Culicoides* and other Diptera in Jasper County, South Carolina. *Mosquito News* 18: 18-21.

Sollai, G., P. Solari, F. Loy, C. Masala, R. Crnjar, A. Liscia. 2010. Morpho-functional identification of abdominal olfactory receptors in the midge *Culicoides imicola. Journal of Comparative Physiology A* 196: 817-824.

Spinelli, G.R. and A. Borkent. 2004. New species of Central American *Culicoides* Latreille (Diptera: Ceratopogonidae) with a synopsis of species from Costa Rica. *Proceedings of the Entomological Society of Washington* 106: 361-395.

Sutcliffe, J.F. and P.D. Deepan. 1988. Anatomy and function of the mouthparts of the biting midge, *Culicoides sanguisuga* (Diptera: Ceratopogonidae). *Journal of Morphology* 198: 353-365.

Swanson, D.A. and P.H. Adler. 2010. Vertical distribution of haematophagous Diptera in temperate forests of the southeastern U.S.A. *Medical and Veterinary Entomology* 24: 182-188.

Swanson, D.A., P.H. Adler, and B. Malmqvist. 2012. Spatial stratification of host-seeking Diptera in boreal forests of northern Europe. *Medical and Veterinary Entomology* 26: 56-62.

Swanson, D.A. and W.L. Grogan, Jr. 2011. A new predaceous midge in *Brachypogon* (*Brachypogon*) from Alabama and Florida, USA and new distribution records for *Brachypogon woodruffi* Spinelli and Grogan (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 113: 531-540.

Szadziewski, R. 1996. Biting midges from Lower Cretaceous amber of Lebanon and Upper Cretaceous Siberian amber of Taimyr (Diptera, Ceratopogonidae). *Studia Dipterologica* 3: 23-86.

Tanner, G.D. and E.C. Turner, Jr. 1974. Vertical activities and host preferences of several *Culicoides* species in a Southwestern Virginia forest. *Mosquito News* 34: 66-70.

Uslu, U. and B. Dik. 2010. Chemical characteristics of breeding sites of *Culicoides* species (Diptera: Ceratopogonidae). *Veterinary Parasitology* 169: 178-184.

Vargas, L. 1953. *Beltranmyia* n. subgen. de *Culicoides* (Insecta: Heleidae). *Revista del Instituto de Salubridad y Enfermedades Tropicales* 13: 33-36.

Vargas, L. 1973. *Wirthomyia*, a new subgenus of *Culicoides* (Diptera: Ceratopogonidae). *Mosquito News* 33: 112-113.

Vitale, G.C., W.W. Wirth, and T.H.G. Aitken. 1981. New species and records of *Culicoides* reared from arboreal habitats in Panama, with a synopsis of the *debilipalpis* group (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 83: 140-159.

Wirth, W.W. 1951. The genus *Culicoides* in Alaska (Diptera: Heleidae). *Annals of the Entomological Society of America* 44: 75-86.

Wirth, W.W. 1977. A new *Culicoides* biting midge from California (Diptera: Ceratopogonidae). *Pan-Pacific Entomologist* 53: 53-55.

Wirth, W.W. and F.S. Blanton. 1967. The North American *Culicoides* of the *guttipennis* group (Diptera: Ceratopogonidae). *Florida Entomologist* 50: 207-232.

Wirth, W.W. and F.S. Blanton. 1969. North American *Culicoides* of the *guttipennis* group (Diptera: Ceratopogonidae). *Florida Entomologist* 52: 207-243.

Wirth, W.W., P.P. Choudhuri, and S.K. Dasgupta. 1985a. Descriptive notes and new Asian distribution in the Chaetophthalmus group of the bloodsucking midge genus Culicoides (Diptera: Ceratopogonidae). *Oriental Insects* 19: 103-109.

Wirth, W.W., A.L. Dyce, and B.V. Peterson. 1985b. An atlas of wing photographs, with a summary of the numerical characters of the Nearctic species of *Culicoides* (Diptera: Ceratopogonidae). *Contributions of the American Entomological Institute* 22: 1-46.

Wirth, W.W. and A.A. Hubert. 1960. Ceratopogonidae (Diptera) reared from cacti, with a review of the *copiosus* group of *Culicoides*. *Annals of the Entomological Society of America* 53: 639-658.

Wirth, W.W. and A.A. Hubert. 1962. The species of *Culicoides* related to *piliferus* Root and Hoffman in Eastern North America (Diptera, Ceratopogonidae). *Annals of the Entomological Society of America* 55: 182-195.

Wirth, W.W. and A.A. Hubert. 1989. The *Culicoides* of southeast Asia (Diptera: Ceratopogonidae). *Memoirs of the American Entomological Institute* 44: 1-508.

Wirth, W.W. and N. Marston. 1968. A method for mounting small insects on microscope slides in Canada balsam. *Annals of the Entomological Society of America* 61: 783-784.

Wirth, W.W. and A.P.A. de Moraes. 1979. New records and new species of biting midges from salt marshes in California and Mexico (Diptera: Ceratopogonidae). *Pan-Pacific Entomologist* 55: 287-298.

Wirth, W.W. and B.A. Mullens. 1992. *Culicoides boydi* (Diptera: Ceratopogonidae): A potential vector of hemorrhagic disease viruses to desert bighorn sheep in southern California. *Journal of Medical Entomology* 29: 1006-1010.

Wirth, W.W. and S. Navai. 1978. Terminology of some antennal sensory organs of *Culicoides* biting midges (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 15: 43-49.

Wirth, W.W. and W.A. Rowley. 1971. A revision of the *palmerae* group of the genus *Culicoides*. *Journal of the Kansas Entomological Society* 44: 153-171.

Wood, D.M. and A. Borkent. 1989. Chapter 114. Phylogeny and classification of the Nematocera, pp. 1333–1370. *In* J. F. McAlpine (Editor) and D.M. Wood. Manual of Nearctic Diptera, Vol. 3. Agriculture Canada Monograph, 32. 248 pp.

Wright, P.J. and C.S. Easton. 1996. Natural incidence of *Lagenidium giganteum* Couch (Oomycetes: Lagenidiales) infecting the biting midge *Culicoides molestus* (Skuse) (Diptera: Ceratopogonidae). *Australian Journal of Entomology* 35: 131-134.

Zimmerman, R.H. and E.C. Turner, Jr. 1983. Host-feeding patterns of *Culicoides* (Diptera: Ceratopogonidae) collected from livestock in Virginia, USA. *Journal of Medical Entomology* 20: 514-519.

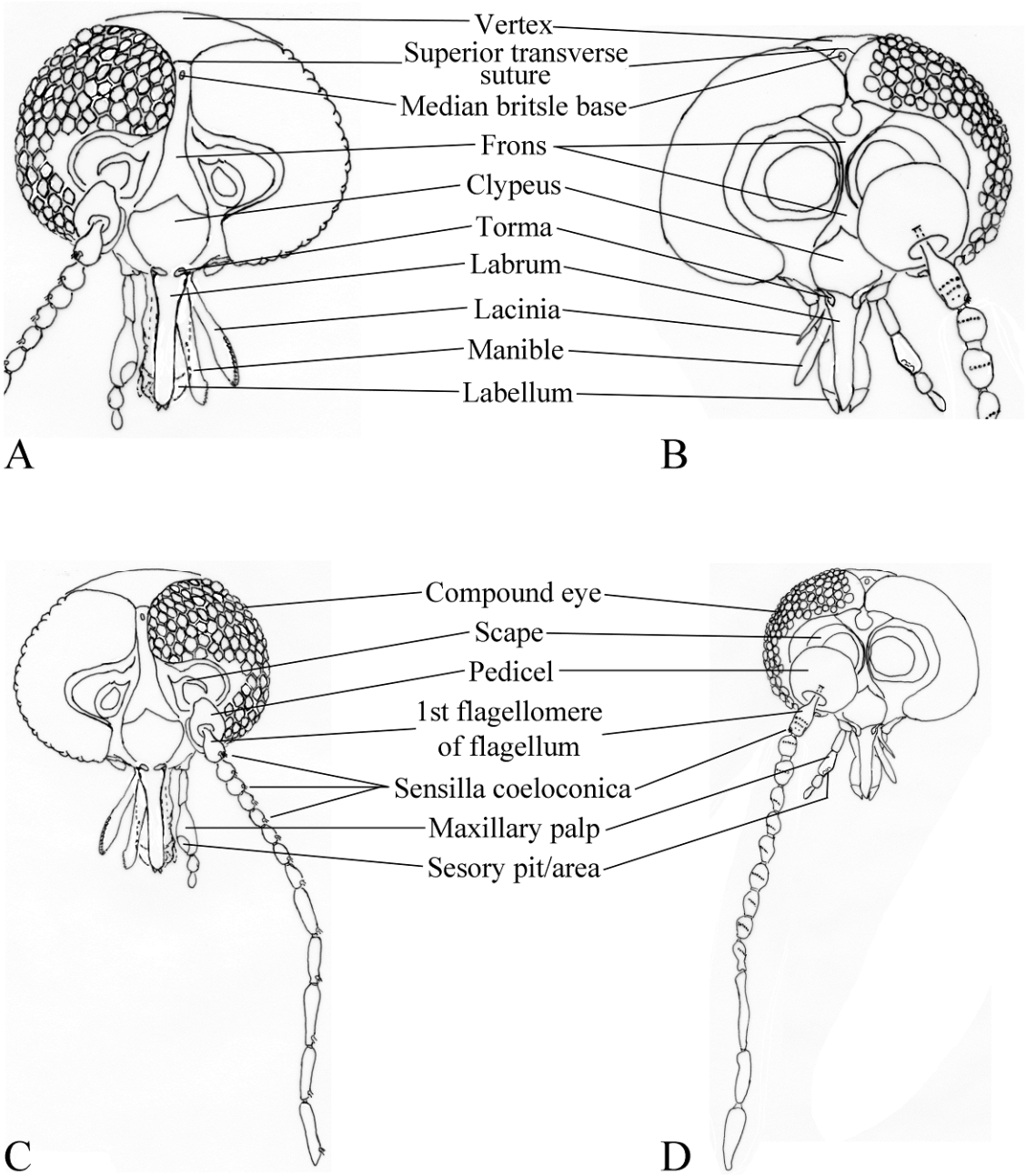


Figure 1.1. Morphology of the head and mouthparts of female and male *Culicoides*. A, C: Female *Culicoides haematopotus* (anterior view); B, D: male *Culicoides biguttatus* with antennal plume removed (anterior view).

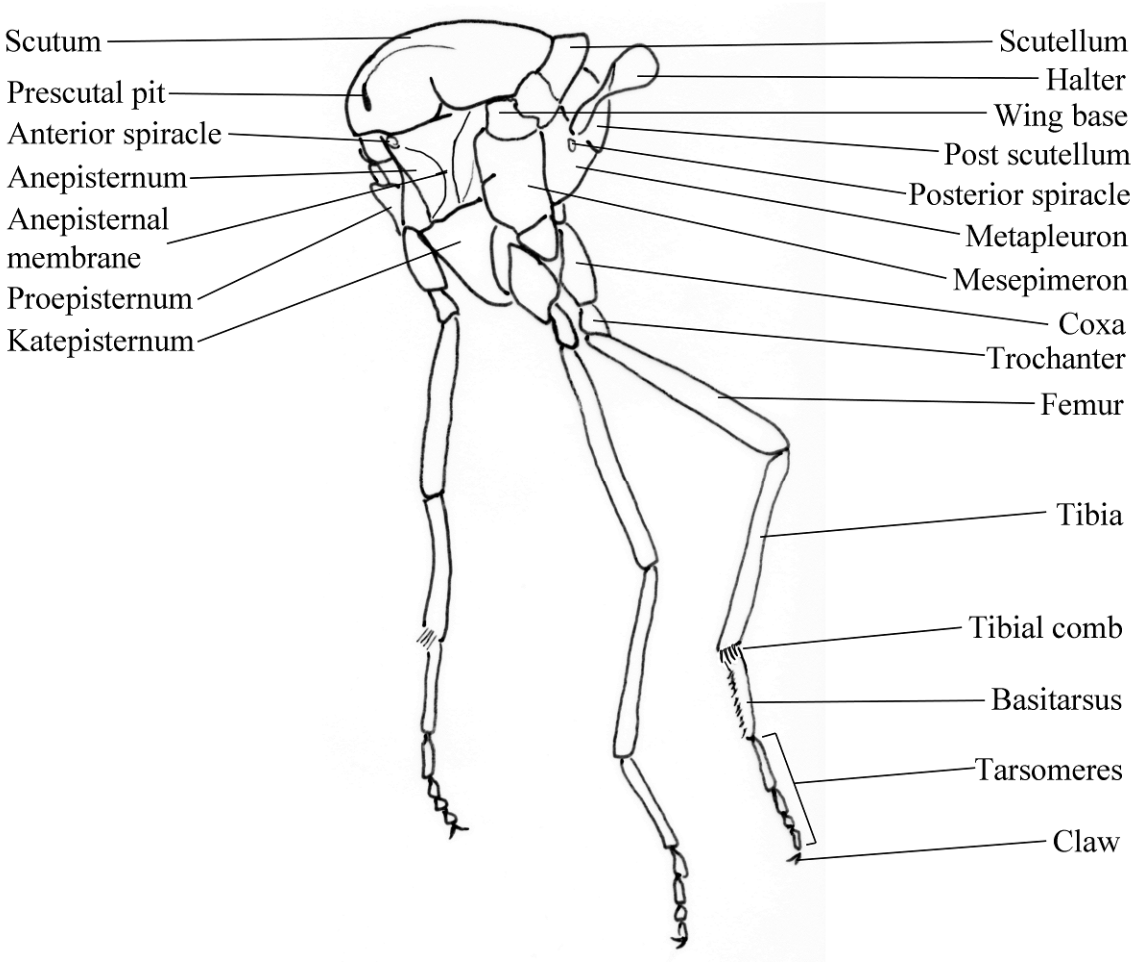


Figure 1.2. Morphology of the thorax and legs of *Culicoides haematopotus* (left lateral view).

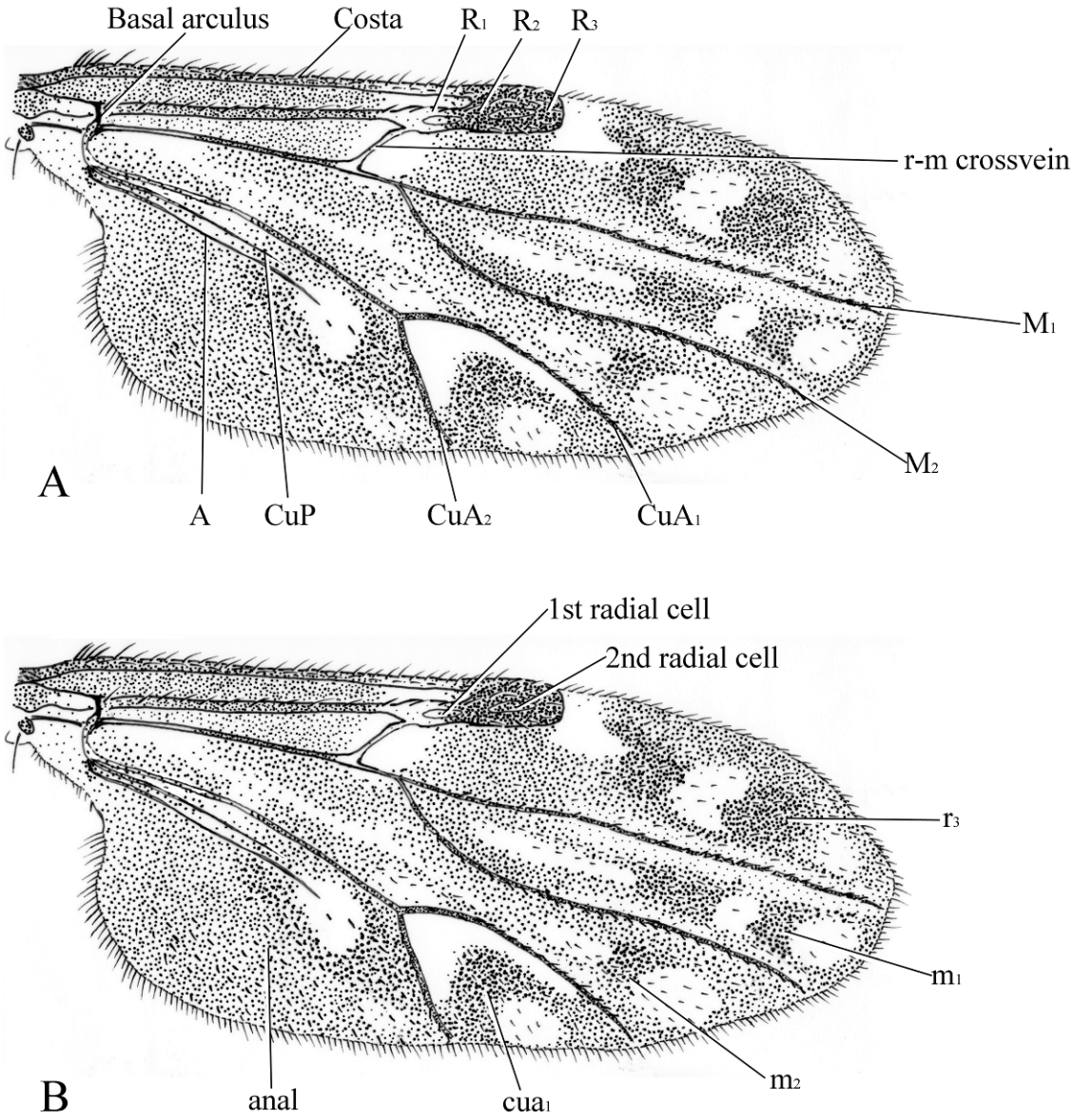


Figure 1.3. Wing of female *Culicoides variipennis*. A: Wing veins; B: Wing cells.

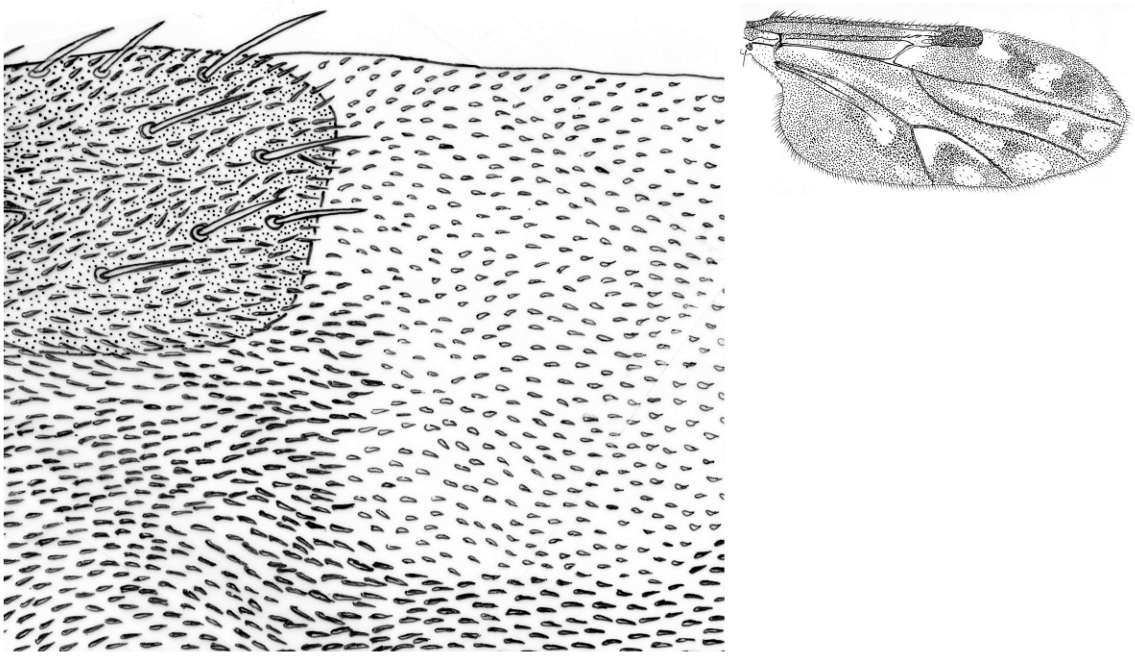


Figure 1.4. Morphology of the wing of female *Culicoides variipennis* magnified to illustrate the light and dark patterns on the wings formed by microtrichia.

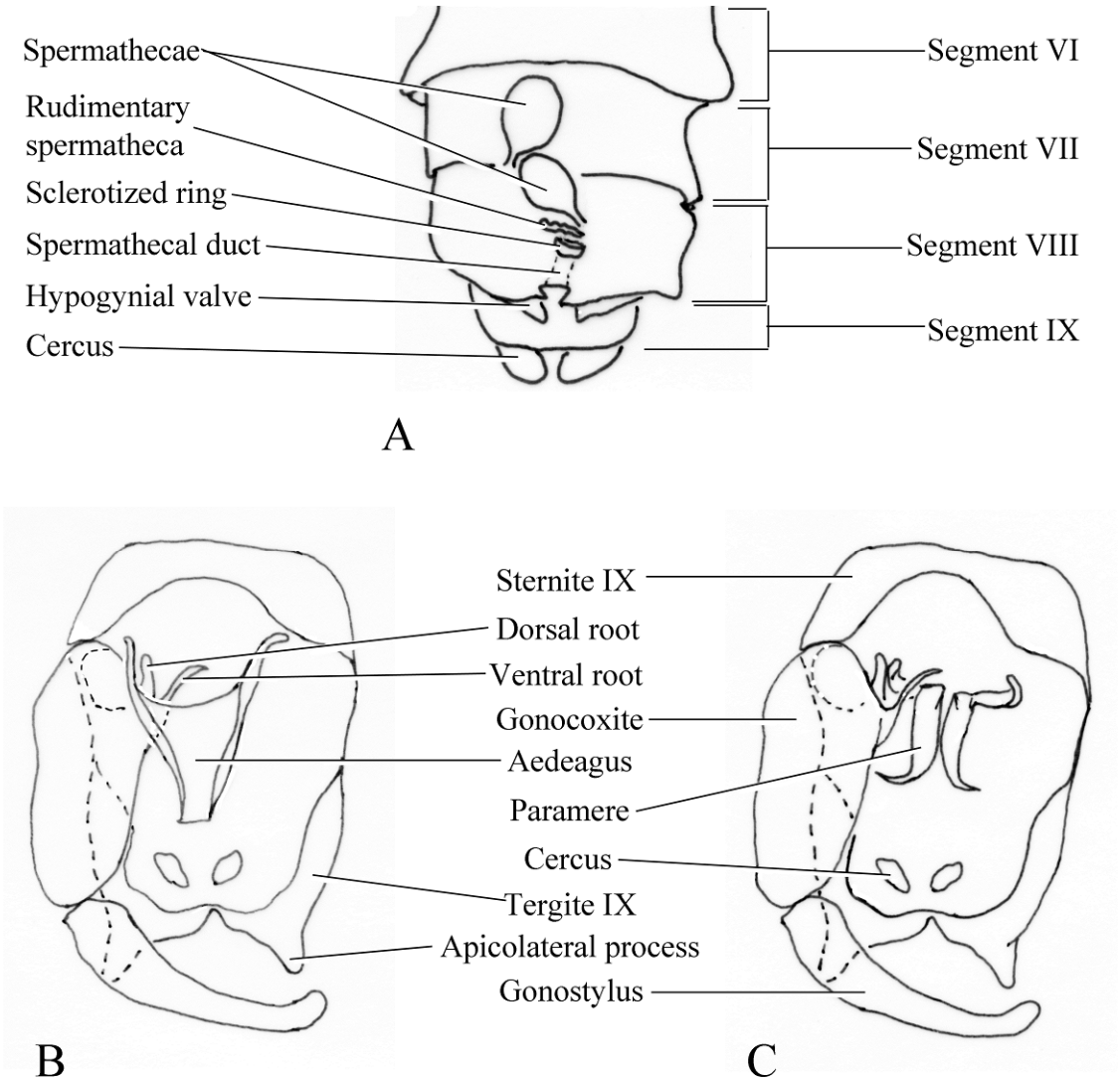


Figure 1.5. Morphology of the terminalia of *Culicoides*. A: female (ventral view), B: male with parameres removed (ventral view), C: male with aedeagus removed (ventral view).

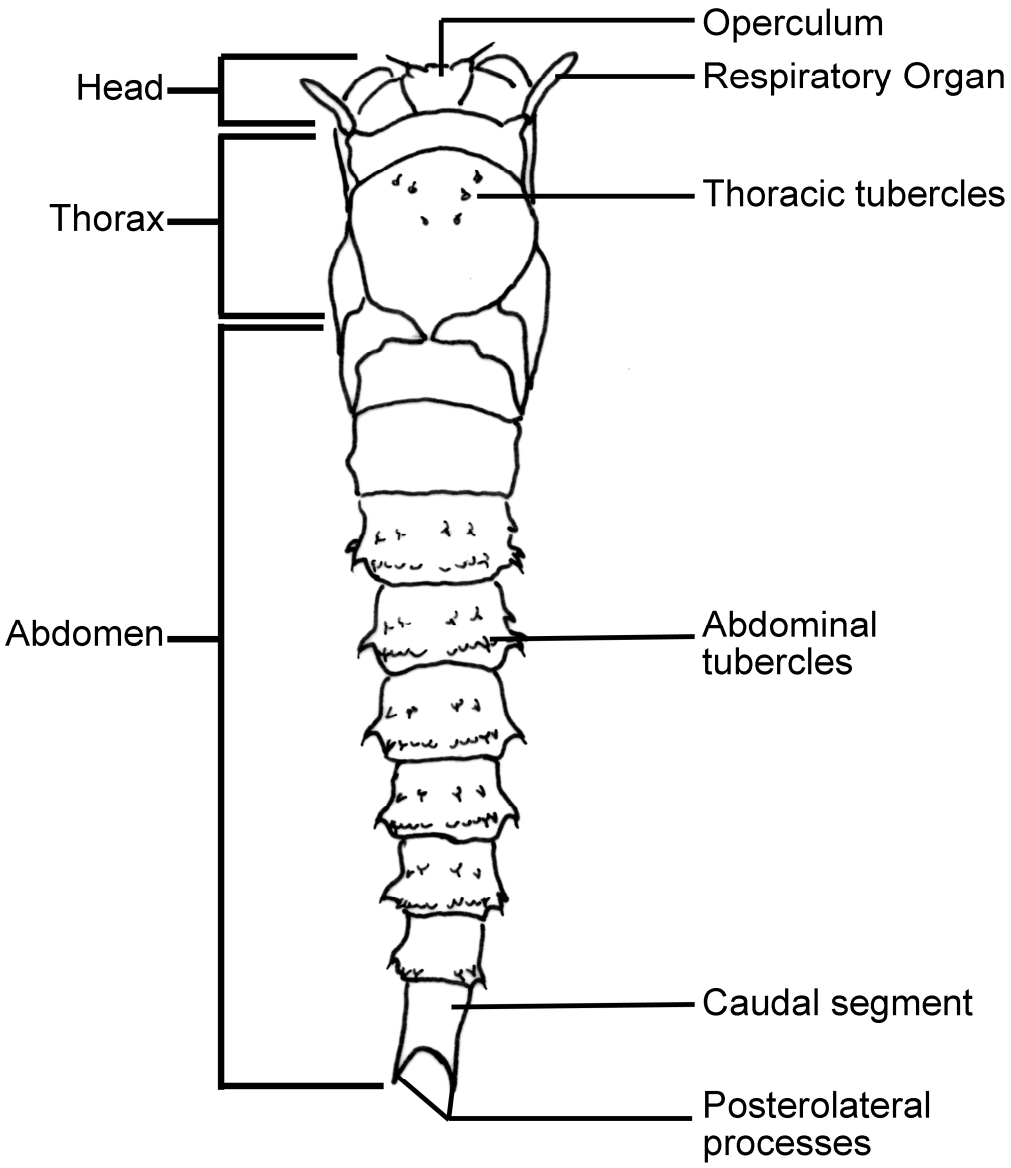


Figure 1.6. General morphology of the pupa of *Culicoides* (dorsal view).

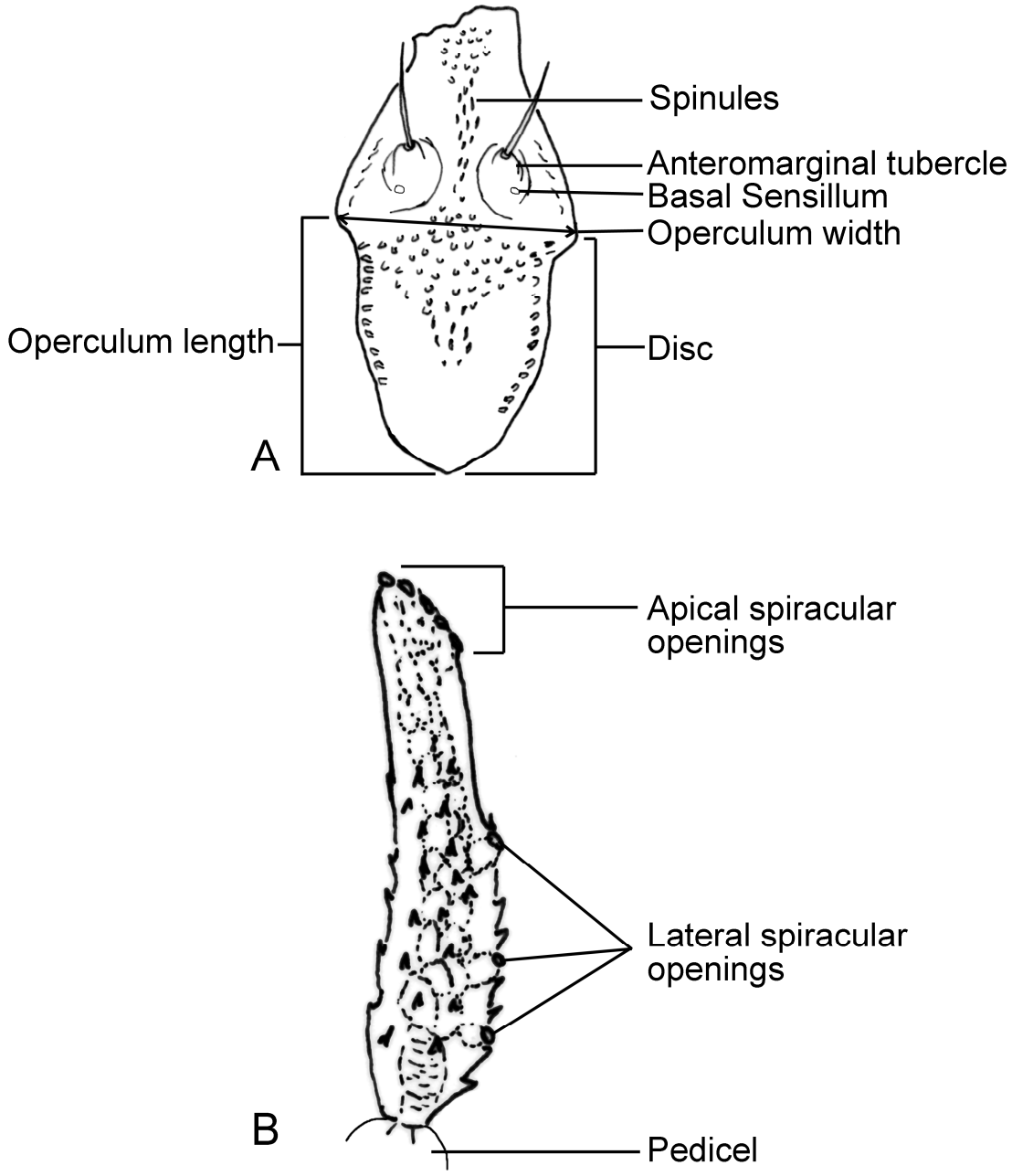


Figure 1.7. Morphology of the pupal operculum and respiratory organ of *Culicoides*. A: operculum (anterodorsal view), B: respiratory organ (lateral view)

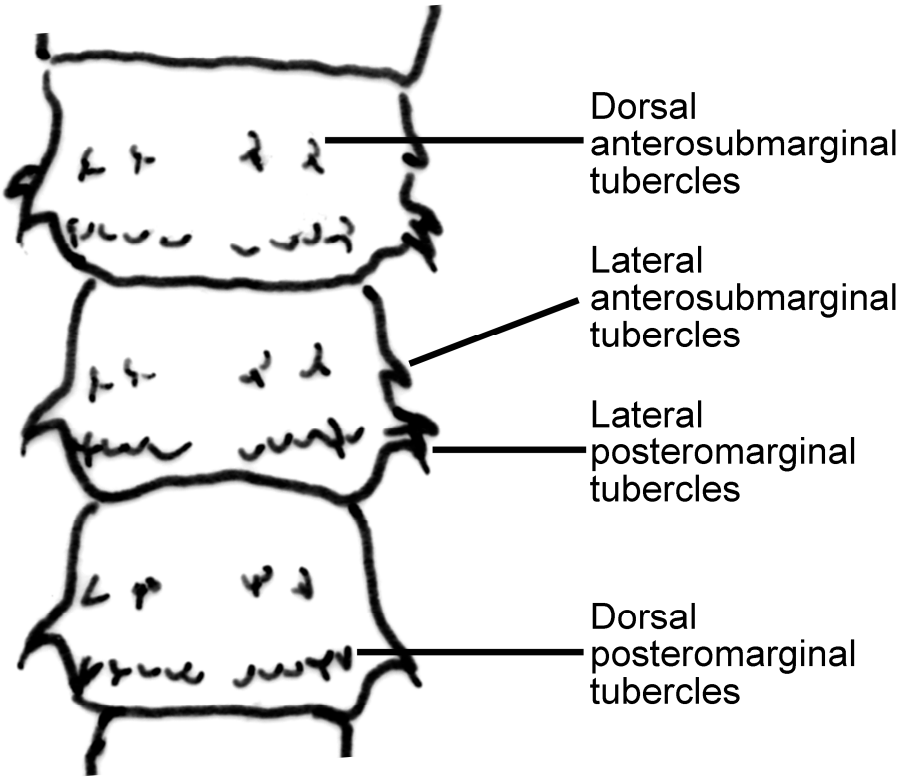


Figure 1.8. Morphology of the abdominal tubercles of segments III-V of a pupa of *Culicoides* (dorsal view).

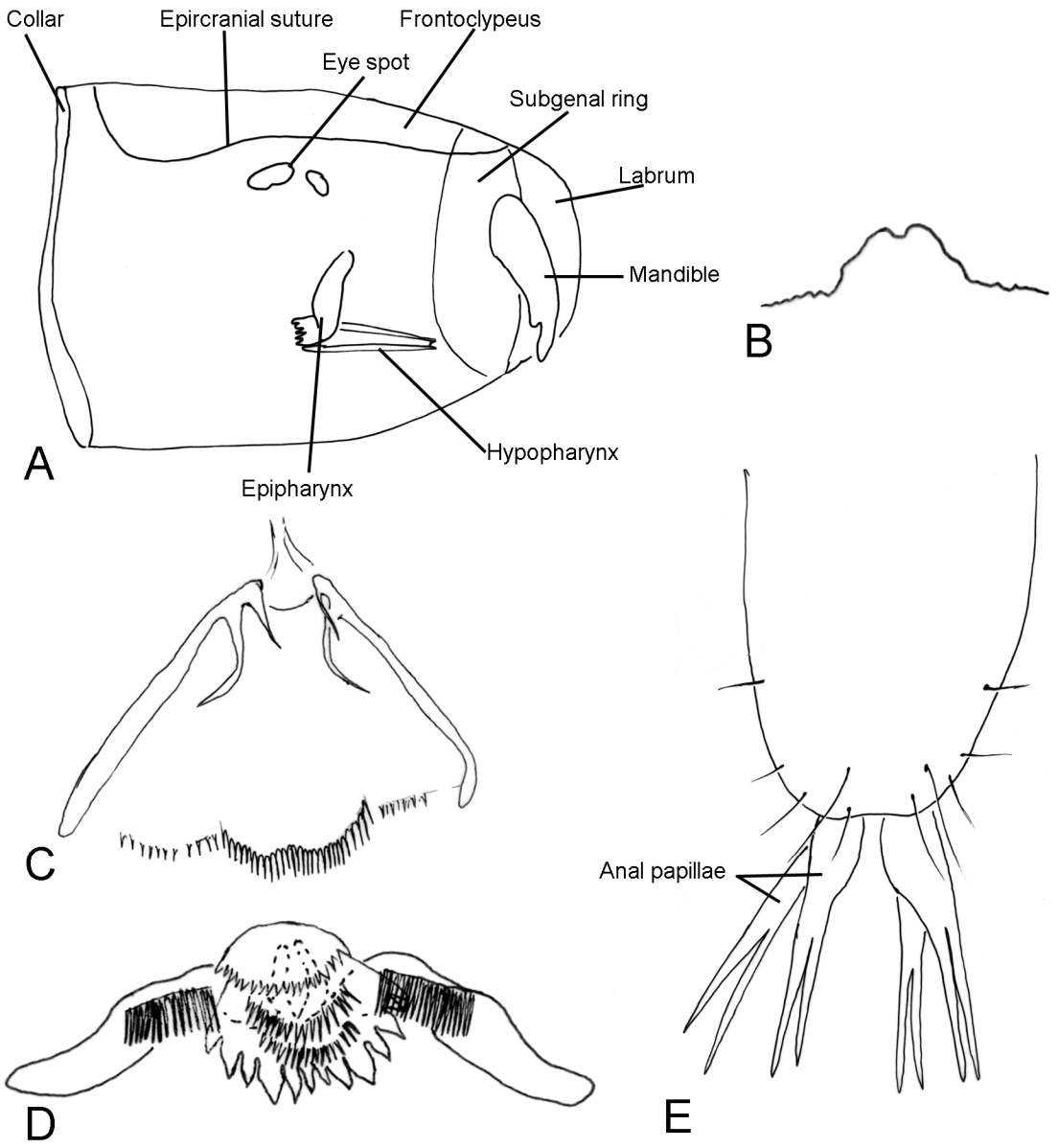


Figure 1.9. Morphology of larval *Culicoides*. A: generalized morphology of the head, B: hypostoma, C: hypopharynx, D: epipharynx, E: caudal segment of abdomen.

CHAPTER TWO

ECOLOGY OF LARVAL *CULICOIDES* IN SOUTH CAROLINA, USA

Introduction

Biting midges of the genus *Culicoides* are species of one of four blood-feeding genera in the family Ceratopogonidae that are vectors of disease agents. Worldwide, species of *Culicoides* have been linked to the transmission of 66 viruses, 15 protozoan species, and 26 filarial-nematode species (Borkent 2005). Some of these disease agents are of significant economic importance; bluetongue virus, epizootic hemorrhagic disease virus, and African horse sickness virus cause large economic losses to livestock producers around the world (Mellor et al. 2000, Bram et al. 2002). The niche space of these insects is largely unexplored. High densities of larvae in certain habitats (Lardeux and Ottenwaelder 1997) could be a significant component of the food web, though the role of *Culicoides* species in food webs has not been assessed.

The emergence of bluetongue virus in southern and central Europe (Mehlhorn et al. 2007) has invigorated interest in the ecology of the genus, but studies have focused more on the adults (Purse et al. 2004, Calvette et al. 2008). The biology of the immature stages represents a major gap in our knowledge of *Culicoides*. Only 19% of the world fauna of *Culicoides* has been described in the larval or pupal stage (Borkent 2012), limiting our knowledge of their ecology. Studies of larval ecology have focused on known vectors (Mullens and Lii 1987, Mullens and Rodriguez 1988, 1992, Schmidtmann et al. 2011), biting pests of humans (Aussel and Linley 1994, Blackwell and King 1997, Blackwell et al. 1994, Kline and Axtell 1977, Kline and Roberts 1982, Kline and Wood

1988, Lardeux and Ottenwaelder 1997, Magnon et al. 1990), and tree-hole species (Kardatzke and Rowley 1971; Kruger et al. 1990; Pappas and Pappas et al. 1990; Pappas et al. 1991). We still know relatively little about the ecology of *Culicoides,* especially the immature stages, and those species not known to directly affect humans or their livestock have received little attention.

My objectives were to improve the larval taxonomy of *Culicoides* by providing a molecular method of identification, and to investigate the larval ecology of *Culicoides* species across ecoregions in South Carolina, USA. The results could improve vector management efforts and aid future ecological studies of the genus. An understanding of the larval ecology also could provide taxonomic and phylogenetic insight for the genus.

Materials and Methods

Study Sites

Eight sites were sampled in the four ecoregions of South Carolina—the coastal plains, sandhills, piedmont, and mountains (Myers, et al. 1986; modified by McCreadie and Adler 1998)—with two sites per ecoregion (Fig. 2.1).

The Clemson Coastal Research and Education Center (32.79 N, 80.07 W) and Huntington Beach State Park (38.52 N, 79.06 W) were selected in the coastal plains. The Coastal Research and Education Center (Fig. 2.1, site A) was 8 km south-southeast of the city of Charleston on 131.5 hectares (http://www.clemson.edu/public/rec/coastal/). Habitats included a mixture of agricultural fields and forested areas (loblolly pine, oaks, tupelo, and sweet gum) interspersed with irrigation ponds and marshes (freshwater and saltwater). Larval collections were predominantly from the salt marsh but also included

freshwater marshes, irrigations ponds, and irrigation ditches (Fig. 2.2A). Huntington Beach State Park (Fig. 2.1, site B) was 32.2 km north of the city of Georgetown in Georgetown County on 1012 hectares (http://www.southcarolinaparks.com/). The park consisted of salt marsh, forest (loblolly pine and oaks), sand beaches, and freshwater ponds. Larval collections were predominantly from the salt marsh surrounding the boat launch (Fig. 2.2B).

Woods Bay State Park (33.95 N, 79.98 W) and Congaree National Park (33.83N, 80.82 W) were selected in the sandhills. Woods Bay State Park (Fig. 2.1, site C) was 32.2 km east of the city of Sumter in Sumter and Clarendon Counties on 643.5 hectares (http://www.southcarolinaparks.com/). The majority of the park was a Carolina bay, a habitat unique to the Mid-Atlantic States. The bay was a cypress-tupelo swamp, with a boardwalk affording access to the interior. Spillover from the bay produced a small stream. Collections were made along the boardwalk, edges of the bay, and the stream (Fig. 2.2C). Congaree National Park (Fig. 2.1, site D) was 20 km southeast of Columbia in Richland County along the banks of the Congaree River. The park was approximately 10,900 hectares, including 4,500 hectares of old growth bottomland hardwood forest (http://www.nps.gov/cong/index.htm). Habitats in the park included cypress and tupelo sloughs, oxbow lakes, hardwood swamps, and upland pine forests. Larval sampling was conducted in a hardwood swamp, along Weston Lake, and in cypress sloughs around Weston Lake (Fig. 2.2D).

Hickory Knob State Resort Park (33.88 N, 82.42 W) and the Clemson Experimental Forest (34.75 N, 82.86 W) were selected from the piedmont ecoregion.

Hickory Knob State Resort Park (Fig. 2.1, site E) was 10 km west of the town of McCormick in McCormick County on 442 hectares along the shores of Strom Thurmond Reservoir, an artificial lake on the Savannah River (http://www.southcarolinaparks.com/). Mixed hardwood and pine forests covered the rolling hillsides and numerous ephemeral and a few permanent streams emptied into the reservoir. Collections were made along the shores of the reservoir and from streams when water was present (Fig. 2.3 E). The Clemson Experimental Forest (Fig. 2.1, site F) was located in Oconee, Pickens, and Anderson Counties and was composed of approximately 7,000 hectares of forests around the town of Clemson (http://www.clemson.edu/cafls/departments/forestry/cef/index.html). Samples were collected in the larger northern tract of forest near Sixmile Creek, adjacent marshes, and the shores of Lake Isaqueenna (Fig 2.3F).

Table Rock State Park (35.03 N, 82.70 W) and Jones Gap State Park (35.13 N, 82.57 W) were selected in the mountains ecoregion. Table Rock State Park (Fig. 2.1, site G) was 15 km north of the town of Pickens in Pickens County on 1,248 hectares (http://www.southcarolinaparks.com/). Two artificial lakes, Pinnacle Lake and Lake Oolenoy, were in the southern portion of the park. Forest habitat was a mix of hardwood trees, pines, and hemlocks. Samples were collected from Pinnacle Lake, Lake Oolenoy, small streams flowing into Pinnacle Lake, and streams along the Carrick Creek nature trail (Fig. 2.3G). Jones Gap State Park (Fig. 2.1, site H) was 12.5 km north-northwest of Marietta in Greenville County on 1,354 hectares in the Blue Ridge Escarpment (http://www.southcarolinaparks.com/). The Middle Saluda River flowed through the park. Forest vegetation was similar to that of Table Rock State Park. Samples were

collected from the banks of the Middle Saluda River and from Cox Camp Creek along Rainbow Falls Trail (Fig. 2.3H).

Larval Sampling and Ecological Measurements

Sites were sampled once every season under permit numbers N-11-08 for Huntington Beach, Hickory Knob, Table Rock, and Jones Gap State Parks; N-07-08 for Woods Bay State Park; and CONG-2009-SCI-0014 for Congaree National Park. Many species of *Culicoides* emerge in the spring months, other species in the summer, and other species have multiple emergences; sampling in every season provided the opportunity to collect a greater number of species. Twelve samples were collected from each site on the same day (unless inclement weather prevented a complete collection of 12 samples). Sampling efforts mirrored the proportions of habitat composition for research sites. For example, the Clemson Coastal Research and Education Center was predominantly salt marsh (60-80%), with a few freshwater habitats (20-40%). Therefore, 7-9 samples were collected from the salt marsh and 3-5 samples from freshwater sources. Sample location within sites was determined by accessibility of the area.

Eleven ecological variables were recorded for each sample, four quantitative and seven categorical. These variables were chosen because they are routinely used in aquatic ecology studies and easy to measure in the field, providing a potential field method to quickly identify breeding habitats of *Culicoides* species. Quantitative variables included temperature (°C), pH, conductivity (µS/cm), and depth (cm). Temperature and pH were measured using an Oyster-10 pH/mV/Temperature meter (Extech Instruments, Nashua, NH). Conductivity was measured using a B-173 Twin Conductivity Meter (Horiba,

Edison, NJ). Categorical variables included ecoregion, season, canopy coverage, surrounding flora, salinity, habitat type, and dominant substrate particle. Categories for ecoregion were coastal plains, sandhills, piedmont, and mountains. Season included winter (22 Dec-20 Mar), spring (21 Mar-21 Jun), summer (22 Jun-21 Sep), and autumn (22 Sep-21 Dec). Canopy coverage was broken into open (no tree coverage), partial (tree coverage on one side), and full (tree coverage on all sides). Surrounding flora was categorized as open (no vegetation), grasses, grasses and trees (grasses with sparse trees, or forest marsh interface), hardwood forest, pine forest, mixed hardwood and cypress forest, mixed hardwood and pine or hemlock forest, and mixed hardwood, pine, and cypress forest. Categories for salinity included freshwater (<0.05% salinity) or saltwater/brackish water (>0.05%). Habitat type was defined as pool (small, ephemeral body of water), pond or lake (large body of water without emergent vegetation), marsh (water with emergent grasses or herbaceous plants), swamp (water with emergent trees), or lotic. Dominant substrate particles were those particles that composed greater than 50% of the sample, and was categorized as living organic (e.g., roots), dead organic (leaf litter, thatch, and other decaying plant matter), silt/clay (substrate not retained in 0.297-mm mesh sieve), sand (substrate retained by 0.297-mm mesh sieve but not 2-mm mesh sieve), and gravel (mineral substrate retained by 2-mm mesh sieve).

Samples were collected from aquatic habitats by inserting a post-hole digger into the substrate as far as the blades could penetrate. If the blades did not penetrate to a depth of 5 cm, that location was not used and a sample was extracted from a different location. Samples were a standard surface area of 132.7 cm^2 but not a standard volume. In

previous studies, a majority of larval *Culicoides* (>87%) were collected in the top 5 cm of substrate (Blackwell and King 1997, Uslu and Dik 2006), therefore, a standardized volume of substrate was not necessary for assessment of presence-absence if a 5-cm deep sample was obtained. Samples were placed in plastic bags and transported to the laboratory. Samples were washed through a 2-mm mesh (10 mesh) sieve and collected in a 0.297-mm mesh (50 mesh) sieve. Larvae were collected by floating the filtrate from the 0.297-mm mesh sieve in a 150% (w/v) sucrose solution. Samples were agitated and examined with a 10X diopter magnifying lamp, and larvae were collected from the surface with forceps for 3 min. Larvae were identified as Ceratopogonidae by the characteristic serpentine-swimming motion. Samples were agitated every 3 min for 30 min or until three consecutive cycles of agitation and collection without ceratopogonid larvae. Specimens were fixed in 95% ethanol.

Adult Collections

Adults were collected on the same dates as larvae, using carbon dioxide-baited ultraviolet Centers for Disease Control and Prevention (CDC) traps. Traps were placed 1.5 and 10.0 m above ground to maximize the species richness (Swanson and Adler 2010, Swanson et al. 2012). Four traps were set per site, two at 1.5 m and two at 10.0 m, using the placement procedure described by Swanson and Adler (2010). Briefly, a fishing line was shot over a branch, using a bow-and-arrow apparatus, which was used to pull a rope over the branch. The rope was attached to the trap and hoisted to the desired height. Traps were baited with approximately 1.0 kg of dry ice above each trap. Traps were run from

approximately 2 h before sunset to 2 h after sunrise. Specimens were placed on dry ice, transported to the laboratory, and fixed in 95% ethanol.

Adult COI Database

Adults were sorted and tentatively identified to species using the photographic wing atlas of Wirth et al. (1985). DNA was isolated from at least one specimen of each morphospecies (Table 2.1), using the Wizard® SV Genomic DNA Purification System (Promega Corporation, Madison, WI). Additional *Culicoides* species and other genera of Ceratopogonidae from Alabama, Illinois, Wisconsin, and Wyoming were used to supplement collections in South Carolina (Table 2.1).Whole midges were placed in extraction solution for 18-24 h and the exoskeleton recovered using flame sterilized forceps, which were placed in 100% ethanol. The rest of the purification followed the manufacturer's protocol except for a final elution volume of 30 µl of nuclease-free water. DNA was stored at -20°C. Exoskeletons were slide mounted by soaking them in 100% clove oil for 15 min and mounting in Canada balsam thinned with clove oil. Morphospecies identifications were confirmed using Blanton and Wirth (1979) and Battle and Turner (1971).

A 523-bp fragment of COI gene was amplified by PCR, using primers C1-J-1718 and C1-N-2191 (Table 2.2) (Dallas et al. 2003). Reactions were run in 20-µl volumes, using the Takara ExTaq Hot Start Version (Takara Bio, Inc., Japan) (2.0 µl of 10x buffer, 1.6 µl of 2.5 mM dNTPs, 0.1 µl of polymerase [5 units/µl], 0.6 µl of each 10 mM primer, 3.0 µl of DNA extract. A 4-µl aliquot of each reaction was subjected to 94°C for 3 min; 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s; and a final extension of

72°C for 10 min. Reactions were checked for amplification by electrophoretic separation on a 1.0% agarose gel run at 5V/cm for 45 min and stained with ethidium bromide. The remaining aliquot of each positive sample was purified using the Wizard SV Gel and PCR Cleanup System (Promega) eluting in 30 µl of nuclease-free water. Purified reactions were submitted for direct Sanger sequencing in both directions using primers C1-J-1718 and C1-N-2191 at the Clemson University Genomics Institute.

Sequence ends were trimmed using Lasergene (DNASTAR, Inc., Madison, WI) at a quality score of 25. Forward and reverse sequences were aligned using MUSCLE (Edgar 2004) in MEGA 5 (Tamura et al. 2011) and assembled into single contigs. For ambiguous base pairs, the trace files were examined manually and the base pair scored according to the trace file with the strongest peak for the base pair in question. Adult contigs were then aligned using MUSCLE and the alignment saved in FASTA format.

Larval Identification

Culicoides larvae were identified as those with all cephalic setae simple (A. Borkent, pers. comm.). Larvae were sorted to morphospecies based on head-capsule shape and color, eye-spot shape, thoracic pigmentation, and the size and number of perianal setae.

DNA was extracted from one larva per morphospecies per substrate sample as described for the adults except for the recovery of the exoskeleton. Initially, whole larvae were used and the exoskeleton recovered from the extraction tube prior to centrifugation or spin column after extraction. This method presented two problems: 1) low recovery of exoskeletons and 2) damage of diagnostic features by centrifugation. To overcome these

problems, the head capsule and thorax were removed and placed in 100% ethanol, and the abdomen used for DNA extraction. PCR conditions followed those for the adults except 4 µl of DNA extract were used as template. Cleaned, positive reactions were directly sequenced using primer C1-J-1718 and trimmed in Lasergene.

Individual larval sequences were submitted to a BLASTn search (Altschul et al. 1997) of the adult COI database via BioEdit (Hall 1999) to infer larval identity. A neighbor joining tree of the larval sequences was constructed in MEGA 5 to corroborate the results of the BLAST search by grouping specimens into phylospecies. Larval sequences were aligned using MUSCLE and a Tamura 3-parameter model with a gamma distribution as the nucleotide substitution model. Sequences that were less than 100 bp or sequences that resulted in gaps of more than two base pairs were excluded from the alignment.

Head capsules were examined after clearing in warm lactic acid, soaking in 100% clove oil for 15 min, and mounting in Canada balsam thinned with clove oil. Associations from BLAST searches were confirmed with morphological descriptions from Murphree and Mullen (1991). Characters for morphological confirmation included the hypostoma, epipharynx, and hypopharynx.

Statistical Analysis

Data were statistically analyzed using multiple logistic regression of presence-absence data for each taxon via the R statistical platform (R Development Core Team 2009). Initial models were fit using all variables. This initial model was subjected to stepwise AIC model selection in forward and reverse directions. The variables selected

by AIC selection were used in the final multiple logistic regression model. Predicted probabilities of taxon presence were calculated for each of the variables retained in the final model.

Results

COI sequences from a total of 78 adult specimens representing 33 species of *Culicoides* and 1 species each of 12 other genera (Table 2.1) were sequenced for the DNA database. Twenty-three of the 39 *Culicoides* native to South Carolina (Borkent and Grogan 2009) were sequenced. *Culicoides bermudensis*, *C. parapiliferus*, and *C. crepuscularis,* which are native species to South Carolina, were sequenced from other locations; thus 2/3 of the previously known fauna of *Culicoides* in South Carolina were represented in the DNA database. An additional 7 *Culicoides* species and 12 species from other ceratopogonid genera also were included in the database. *Culicoides chewaclae* Glick and Mullen, 1983, and *C. denticulatus* Wirth and Hubert, 1962, were collected in Congaree National Park and *C. juddi* Cochrane, 1974, in the Clemson Coastal Research and Education Center, representing new state records, bringing the total number of species in the state to 42 (Borkent and Grogan 2009). These specimens were not represented in the DNA database.

A total of 537 substrate samples was collected from the eight sites: 79 from the Clemson Coastal Research and Education Center, 51 from Huntington Beach State Park, 82 from Woods Bay State Park, 80 from Congaree National Park, 45 from Hickory Knob State Park, 96 from the Clemson Experimental Forest, 48 from Table Rock State Park, and 56 from Jones Gap State Park (number of collections among sites due to collecting

trips interrupted by inclement weather). BLAST searches of the database identified 13 taxa to species (Table 2.3). The neighbor joining tree resulted in 35 groups of specimens representing putative species, 19 of which were determined to be non-*Culicoides,* based on cephalic chaetotaxy (Table 2.3, Figure 2.4). In total, 15 taxa of *Culicoides* were identified (Table 2.4): 11 to species level, 1 to species complex, and 3 to morphospecies that might represent new species of *Culicoides.* (Two clades of *C. piliferus* group were grouped together).

Logistic regression was run on 12 of the 15 taxa. (*Culicoides biguttatus*, *C. obsoletus*, and *C. piliferus* group were not analyzed due to small sample sizes). Of the 12 taxa analyzed, the algorithm converged on a model for two taxa: *C. furens* and *C. hollensis*. For the other 10 taxa, the algorithm did not converge on a model.

For *C. furens*, variables retained by stepwise AIC selection included Depth, Conductivity, Season, Surrounding Flora, Dominant Substrate Particle, and Salinity. Of these, Dominant Substrate Particle was significant at an alpha of 0.05 (p=0.0339, z-value = 2.122, df=115) and Season at an alpha of 0.10 (p=0.0798, z-value=1.752, df=115). Samples with Dominant Substrate Particles of dead organic matter were significantly more likely to have larval *C. furens* (Fig. 2.5E). Larval *C. furens* were also more likely to be collected in the spring (Fig. 2.5C). Although not significant, shallower depths (Fig. 2.5A), higher conductivity (Fig. 2.5B), grassy areas (*Spartina alterniflora* Loisel., 1807) and open areas (Fig. 2.5D), and brackish/saltwater (Fig. 2.5F) were more likely to yield larvae of *C. furens*.

For *C. hollensis*, variables retained in the model included Depth, Conductivity, Season, Ecoregion, and Salinity. Season was a significant predictor at an alpha of 0.05, with larvae significantly less likely to be collected in spring (p=0.0147, z-value= -2.440, df=121) and autumn (p=0.0045, z-value=-2.843, df=121) (Fig. 2.6C). Although not significant, greater depths (2.6A) and higher conductivity (2.6B) were more likely to yield larval *C. hollensis*. Ecoregion (2.6D) and Salinity (2.6E) provided limited predictive power.

Culicoides haematopotus was the second most frequently identified species, but the logistic regression did not converge on a model. Closer examination of the neighbor-joining tree revealed seven potential groups within the taxon. A maximum likelihood tree using a Kimura 2-parameter model and all codon positions was computed in MEGA 5 for all *C. haematopotus* sequences. *Culicoides furens*, *Atrichopogon* sp., and *Forcipomyia glauca* were used as outgroups. Six clades (bootstrap support ≥69) were found in the resulting tree (Fig. 2.7). Groups h3, h4, h5, h6, and h7 were used in a hierarchical cluster analysis to determine if groups were ecologically distinct. A Euclidean distance matrix was calculated and the hierarchical cluster analysis performed using Ward, Single, and Average clustering methods in the vegan package of R. All methods converged on similar clustering patterns, with groups h3, h4, and h5 clustering and groups h6 and h7 forming another cluster but also occurring in the h3+h4+h5 cluster (Fig 2.8). Groups h3, h4, and h5 were merged into one group (h3); h6 and h7 were merged into a single group (h6). These new groups were used in the multiple logistic regression. For the h3 group, Depth (p=0.0345, z-value= -2.114, df=469) and Surrounding Flora (p=0.0447, z-value=2.008,

df=469) were statistically significant in the initial model, but the stepwise AIC selection failed to converge on a simpler model. Larvae of group h3 were more likely to be found in substrate at the water line and associated with hardwood forests. For group h6, Temperature, Depth, Conductivity, Ecoregion, Canopy Coverage, Surrounding Flora, and Dominant Substrate Particle were retained in the stepwise AIC selection. Depth (p=0.0127, z-value= -2.491, df=498) and Ecoregion (p=0.0287, z-value= -2.187, df=498) were significant predictors. The probability of collecting h6 larvae increased with lower depths and in the Coastal Plains ecoregion.

Discussion

Knowledge of the larval ecology of *Culicoides* comes largely from accounts of rearing specimens from various aquatic or semi-aquatic substrates. These studies offer a snapshot of the ecology of larval *Culicoides* species but provide little predictive power. Ecological studies of adult *Culicoides* have produced predictive models of adult distribution (Purse et al. 2004; Calvette et al. 2008). Adding ecological data on larval habitats can enhance adult-only models and improve ecological understanding of *Culicoides*.

The difficulty of identifying larval Ceratopogonidae has hindered ecological study of the family. Identification to subfamily can be accomplished with relative ease, but identification to genus and species is more challenging. The presence of only simple setae on the larval head capsule is useful for distinguishing *Culicoides* from other Ceratopogoninae when the setae are not damaged. However, with species T, this character is ambiguous. Some larvae of species T exhibited a compound seta on one side

of the head, while the corresponding seta on the other side was simple. Species T might belong to a different genus, but the larvae share a high sequence identity with an isolate of *C. stellifer* A6 (96-99%). The usefulness of this character to identify larvae needs further study.

DNA barcoding techniques could serve as a valuable tool for ecological and taxonomic studies of ceratopogonid larvae. This method did not require larvae to be fourth instars or to be reared to adults for identification. When used with a non-destructive DNA extraction, this method can be coupled with larval morphology to confirm identifications and associate and describe unknown life stages. The utility of this method was shown with the first associations of larval *C. parapiliferus* Wirth and Blanton, 1974, and *Echinohelea lanei* Wirth, 1951, with their respective adults. This method is dependent on the quality of the DNA database (e.g., number of species, number of sequences, quality of sequences), posing a current limitation of this method. Increasing the taxonomic and geographic representation of species in the DNA database will improve the capabilities of this method and facilitate association of undescribed immature stages with the adults.

The logistic regression models converged upon for *C. furens* and *C. hollensis* are consistent with previous studies. Larval *C. furens* are more likely to be collected at lower depths, higher salinity, in substrates rich with organic matter, and during the spring months. In Florida, significantly more adults were collected in emergence traps associated with short *Spartina alterniflora* (Kline and Axtell 1977) and with black mangrove or mixed red and black mangrove stands (Kline and Roberts 1982). These

habitats were inundated with tides for shorter periods of time, approximately 3 h per day for short stands of *S. alterniflora* (Kline and Axtell 1977), which would correspond to lesser depths in my study. *Culicoides furens* was collected predominantly from saltwater environments but has been collected from freshwater habitats (Rogers 1962). One larva of *C. furens* was identified via molecular and morphological methods from Hickory Knob State Park approximately 250 km from the coast; 2 other specimens from Woods Bay were identified molecularly as *C. furens* but not morphologically. In 2007, a single adult female was collected in Columbia, South Carolina, approximately 170 km from the coast (Nelder, et al. 2010). *Culicoides furens* occasionally might disperse inland following larger rivers (Savannah River for Hickory Knob State Park and Congaree River for Columbia), or these collection records could be the result of human transport (e.g., contaminated collecting equipment). The high likelihood of collecting larval *C. furens* in the spring would correspond with the emergence of adults. Adult *C. furens* begin to emerge in April in Florida (Kline 1986) and in May in North Carolina (Kline and Axtell 1976).

Larvae of *C. hollensis* are more likely to be collected in the winter and summer. These peaks in larval collection precede peak abundances of adults in the spring and autumn (Kline and Axtell 1976). Depth was retained in the logistic regression model, but was not significant. Larvae of this species occur more frequently in stands of tall *S. alterniflora* where time of inundation ranges from approximately 4.5 to 8.5 h (Kline and Axtell 1977) and would correspond to greater depths. My collections were restricted to the margins of salt marshes with more stands of short *S. alterniflora* and shallower

depths. Increased sampling from stands of tall *S. alterniflora* is needed to further evaluate the significance of depth. *Culicoides hollensis* inhabits saltmarshes in coastal areas; thus, ecoregion and salinity were predicted to be significant factors, but these factors were not significant in the logistic regression model and did not have high predicted probabilities (<7% for ecoregion, <1% for salinity). The lack of significance of ecoregion and salinity could be an artifact of a large number of absences in saline and coastal habitats. Much like depth, the importance of these factors can be tested further with sampling from known habitats of *C. hollensis.*

The initial logistic regression for each species only converged on models for two of the twelve species tested. One of the reasons for this lack of convergence could be due to the small sample sizes of the species of *Culicoides* collected. Only four species were collected at frequencies of greater than 4% when all sites were pooled, with many species exhibiting greater frequency for specific sites or ecoregions (Table 2.4). Another explanation is that the ecological variables chosen to assess the distribution of *Culicoides* were not adequate predictors of larval presence-absence. Variables that account for various soil characteristics (e.g., organic content, mineral composition) might be better predictors of larval presence absence. Measuring the variables as continuous rather than categorical variables could help the analyses converge on models. Another factor that might cause the logistic regression not to converge on a model is the presence of cryptic species or ecologically variant sub populations. The *C. bickleyi* complex was the third most frequently collected taxon, but no model was converged on for this taxon. The morphology of the specimens of the *C. bickleyi* complex was highly variable, indicating

the presence of more than one species. The failure of the logistic regression due to cryptic species or ecologically variant sub populations was observed for *Culicoides haematopotus* Malloch, 1915.

Culicoides haematopotus is a widespread species ranging from British Columbia to Nova Scotia south throughout the USA into Mexico and Honduras (Borkent and Grogan 2009). This species has been collected from multiple habitats including cypress sloughs, pond margins, stream margins, ditches, and rain pools (Blanton and Wirth 1979). The wide geographic and habitat range suggests that *C. haematopotus* could be a species complex, which might explain the lack of convergence in the logistic regression model. The neighbor-joining analysis of the larval COI sequences supported this hypothesis. The ecological differences among the neighbor-joining clusters (h3+h4+h5 and h6+h7), further supported the hypothesis of a species complex. *Culicoides stellifer*, another species ranging throughout most of the USA and eastern Canada (Borkent and Grogan 2009), consists of genetically distinct groups. Five larvae share high sequence identity with *C. stellifer* A78 and match morphological descriptions of larval *C. stellifer*. Specimens of species T share high sequence identify with *C. stellifer* A6 but are morphologically distinct from larval *C. stellifer*. Specimens of species T are cluster far from confirmed larvae of *C. stellifer* (Fig. 2.7). These data support a species-complex hypothesis for *C. stellifer*. Further analyses using specimens from other geographic areas and additional loci coupled with ecological analysis could reveal more cryptic species.

My study of South Carolina fauna revealed 2 potential species complexes, 3 previously undescribed larvae, and 2 new distribution records. The ecological data

collected in this study will help other researchers in collecting larvae from habitats by providing models for predicting larval presence and absence. Future studies that include other geographic areas and quantify larvae in substrate samples can further enhance our knowledge of the immature stages and the genus.

References

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402.

Battle, F.V. and E.C. Turner, Jr. 1971. The insects of Virginia: No. 3. A systematic review of the genus *Culicoides* (Diptera: Ceratopogonidae) of Virginia with a geographic catalog of the species occurring in the eastern United States north of Florida. Virginia Polytechnic Institute and State University, Research Division Bulletin 44. 129 pp.

Blackwell, A. and F.C. King. 1997. The vertical distribution of *Culicoides impunctatus* larvae. *Medical and Veterinary Entomology* 11: 45-48.

Blanton, F.S. and W.W. Wirth. 1979. The sand flies (*Culicoides*) of Florida (Diptera: Ceratopogonidae). Arthropods of Florida and Neighboring Land Areas. Volume 10. Florida Department of Consumer Services, Gainesville, Florida, USA. 204 pp.

Borkent, A. 2005. The biting midges, the Ceratopogonidae (Diptera), pp. 113-126. In, W.C. Marquardt, W.C. Black IV, S. Higgs, J.E. Freier, A.A. James, H.H. Hagedorn, B. Kondratieff, J. Hemingway, C.G. Moore (Editors). Biology of Disease Vectors, 2nd Ed. Elsevier Academic Press, New York. 785 pp.

Borkent, A. 2012. The subgeneric classification of *Culicoides* – thoughts and a warning. Illinois Natural History Museum (http://www.inhs.illinois.edu/research/FLYTREE/CulicoidesSubgenera.pdf). [accessed 4 June 2012].

Borkent, A. and W.L. Grogan, Jr. 2009. Catalog of the New World biting midges north of Mexico (Diptera: Ceratopogonidae). *Zootaxa* 2273: 1-48.

Bram, R.A., J.E. George, R.E. Reichard, W.J. Tabachnick. 2002. Threat of foreign arthropod-borne pathogens to livestock in the United States. *Journal of Medical Entomology* 39: 405-416.

Calvette, C., R. Estrada, M.A. Miranda, D. Borras, J.H. Calvo, J. Lucientes. 2008. Modeling the distributions and spatial coincidence of bluetongue vectors *Culicoides imicola* and the *Culicoides obsoletus* group throughout the Iberian Peninsula. *Medical and Veterinary Entomology* 22: 124-134.

Dallas, J.F., R.H. Cruickshank, Y.-M. Linton, D.V. Nolan, M. Patakakis, Y. Braverman, R. Capela, I. Pena, R. Meiswinkel, M.D. Ortega, M. Baylis, P.S. Mellor, A.J. Mordue (Luntz). 2003. Phylogenetic status and matrilineal structure of the biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. *Medical and Veterinary Entomology* 17: 379-387.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792-1797.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Kline, D.L. 1986. Seasonal abundance of adult *Culicoides* spp. (Diptera: Ceratopogonidae) in a salt marsh in Florida, USA. *Journal of Medical Entomology* 23: 16-22.

Kline, D.L. and R.C. Axtell. 1976. Salt marsh *Culicoides* (Diptera: Ceratopogonidae): species, seasonal abundance and comparisons of trapping methods. *Mosquito News* 36: 1-10.

Kline, D.L. and R.C. Axtell. 1977. Distribution of *Culicoides hollensis*, *C. furens*, and *C. bermudensis* in relation to plant cover in a North Carolina salt marsh (Diptera: Ceratopogonidae). *Journal of Medical Entomology* 13: 545-552.

Kline, D.L. and R.H. Roberts. 1982. Daily and seasonal abundance of *Culicoides* spp. biting midges (Diptera: Ceratopogonidae) in selected mangrove areas in Lee County, Florida. *Florida Entomologist* 1965: 126-135.

McCreadie, J.W. and P.H. Adler. 1998. Scale, time, space, and predictability: species distributions of preimaginal black flies (Diptera: Simuliidae). *Oecologia* 114: 79-92.

Mehlhorn, H., V. Walldorf, S. Klimpel, B. Jahn, F. Jaeger; J. Eschweiler, B. Hoffmann, and M. Beer. 2007. First occurrence of *Culicoides obsoletus*-transmitted bluetongue virus epidemic in Central Europe. *Parasitology Research* 101: 219-228.

Mellor, P.S., J. Boorman, and M. Baylis. 2000. *Culicoides* biting midges: Their role as arbovirus vectors. *Annual Review of Entomology* 45: 307-340.

Myers, R.K., R. Zahner and S.M. Jones. 1986. *Forest habitat regions of South Carolina from Landsat imagery*. Forest Research Series 42, Clemson University, Clemson, South Carolina.

Nelder, M.P., D.A. Swanson, P.H. Adler, and W.L. Grogan, Jr. 2010. Biting midges of the genus *Culicoides* in South Carolina zoos. *Journal of Insect Science* 10: 1-9.

Purse, B.V., A.J. Tatem, S. Caracappa, D.J. Rogers, P.S. Mellor, M. Baylis, A. Torina. 2004. Modeling the distributions of *Culicoides* bluetongue virus vectors in Sicily in relation to satellite-derived climate variables. *Medical and Veterinary Entomology* 18: 90-101

R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org [Accessed 16 January 2009].

Rogers, A.J. 1962. Effects of impounding and filling on the production of sand flies (*Culicoides*) in Florida salt marshes. *Journal of Economic Entomology* 55: 521-527.

Swanson, D.A. and P.H. Adler. 2010. Vertical distribution of haematophagous Diptera in temperate forests of the southeastern U.S.A. *Medical and Veterinary Entomology* 24: 182-188.

Swanson, D.A., P.H. Adler, and B. Malmqvist. 2012. Spatial stratification of host-seeking Diptera in boreal forests of northern Europe. *Medical and Veterinary Entomology* 26: 56-62.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731-2739.

Uslu, U. and B. Dik. 2006. Vertical distribution of *Culicoides* larvae and pupae. *Medical and Veterinary Entomology* 20: 350-352.

Wirth, W.W., A.L. Dyce, and B.V. Peterson. 1985. An atlas of wing photographs, with a summary of the numerical characters of the Nearctic species of *Culicoides* (Diptera: Ceratopogonidae). *Contributions of the American Entomological Institute* 22: 1-46.

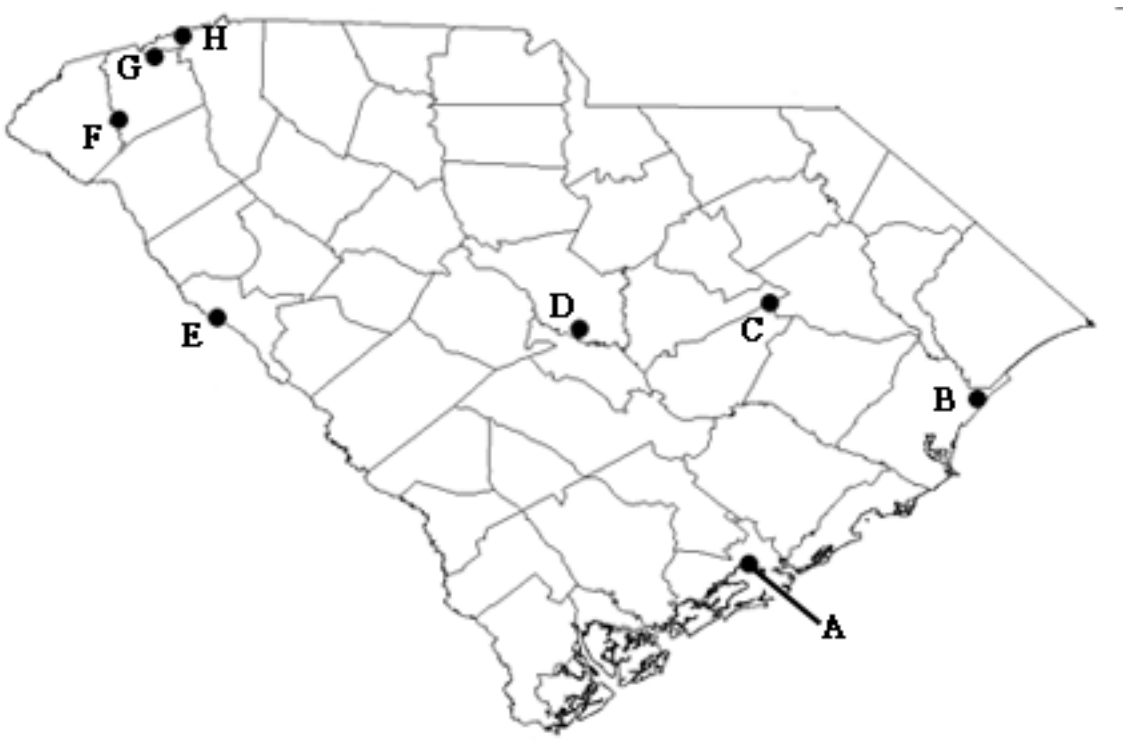


Figure 2.1. Collection sites in South Carolina. A: Clemson Coastal Research and Education Center, B: Huntington Beach State Park, C: Woods Bay State Park, D: Congaree National Park, E: Hickory Knob State Resort Park, F: Clemson Experimental Forest, G: Table Rock State Park, H: Jones Gap State Park.

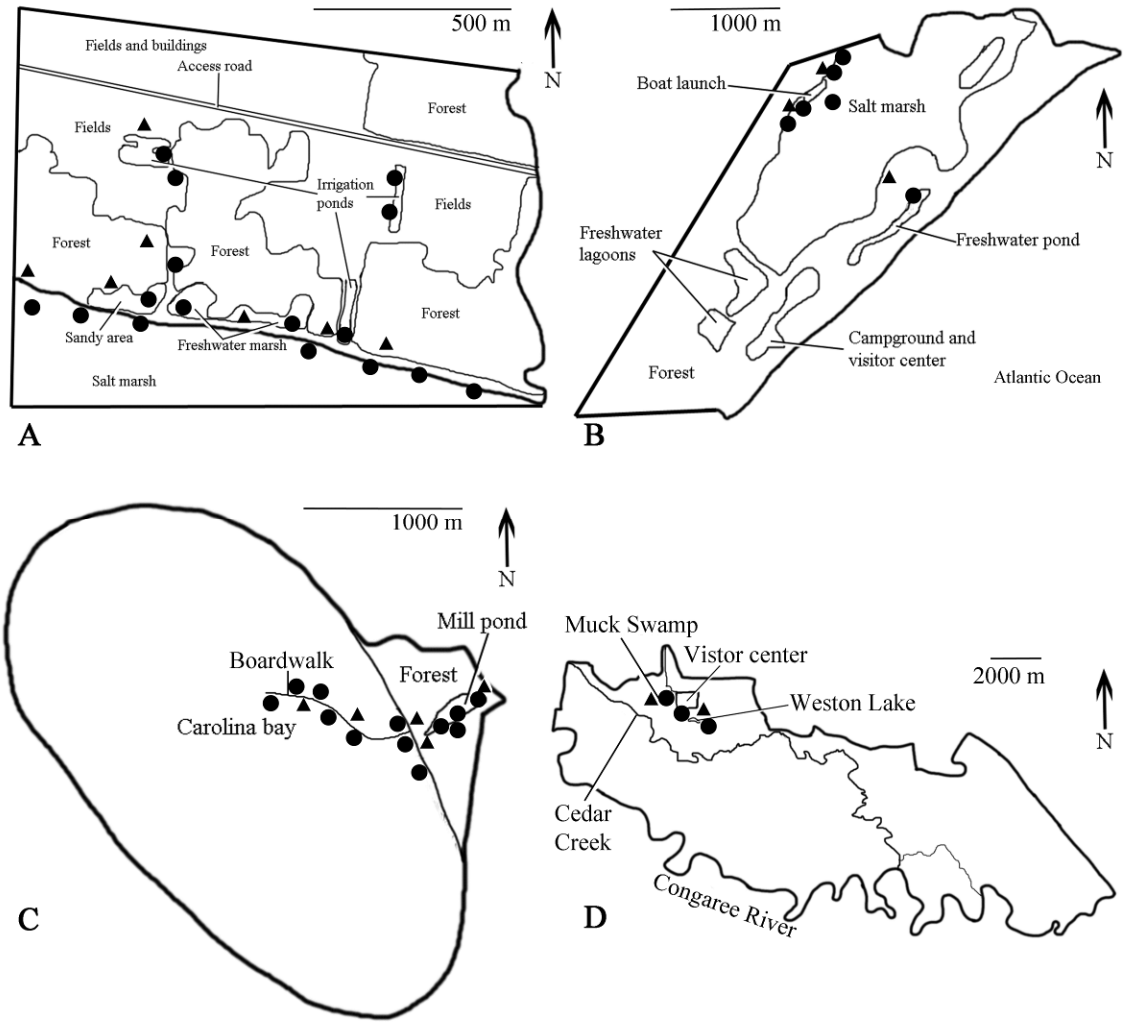


Figure 2.2. Research sites with general locations of larval and adult sampling. A: Clemson Coastal Research and Education Center, B: Huntington Beach State Park, C: Woods Bay State Park, D: Congaree National Park. Circles (●) represent larval sampling locations and triangles (▲) represent adult sampling locations. The number of symbols is not indicative of sampling effort.

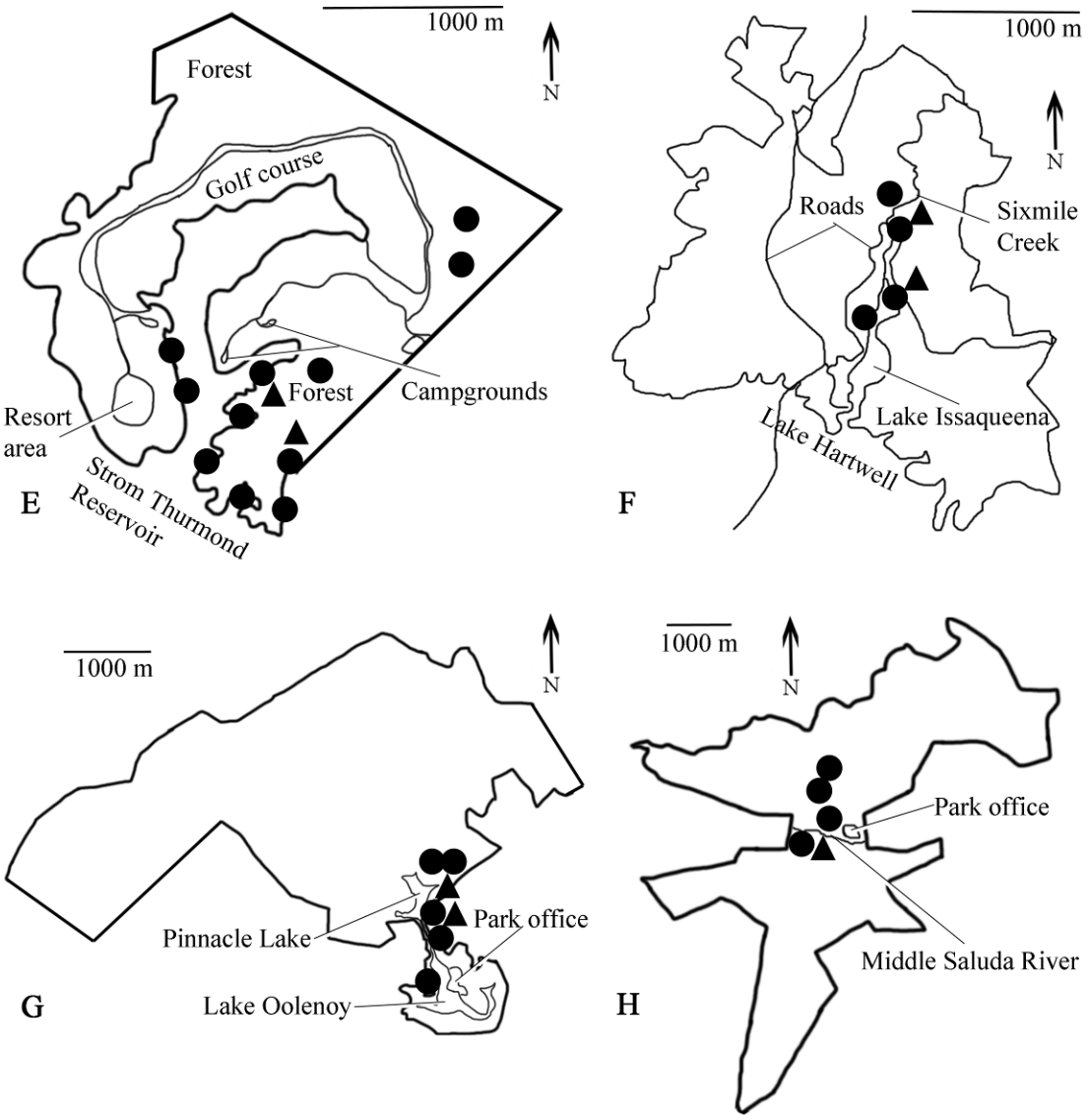


Figure 2.3. Research sites with general locations of larval and adult sampling. E: Hickory Knob State Resort Park, F: Clemson Experimental Forest, G: Table Rock State Park, H: Jones Gap State Park. Circles (●) represent larval sampling locations and triangles (▲) represent adult sampling locations. The number of symbols is not indicative of sampling effort.

Table 2.1. Species, identification codes, collection locations, and dates for adults used to create COI database for identification of larval *Culicoides*.

Taxon	Code	Location	Date
Culicoides haematopotus	A1	SC: Pickens Co. Clemson Exp. Forest	12 Aug 2008
Culicoides stellifer	A6	SC: Pickens Co. Clemson Exp. Forest	12 Aug 2008
Culicoides obsoletus	A7	SC: Pickens Co. Clemson Exp. Forest	3 Nov 2008
Culicoides spinosus	A9	SC: Pickens Co. Clemson Exp. Forest	8 May 2007
Culicoides biguttatus	A10	SC: Pickens Co. Clemson Exp. Forest	8 May 2007
Culicoides baueri	A11	SC: Pickens Co. Clemson Exp. Forest	5 Jun 2008
Culicoides snowi	A12	SC: Pickens Co. Clemson Exp. Forest	30 Apr 2008
Culicoides spinosus	A32	SC: Pickens Co. Clemson Exp. Forest	8 May 2007
Culicoides venustus	A34	SC: Pickens Co. Clemson Exp. Forest	14 Jul 09
Culicoides guttipennis	A36	SC: Pickens Co. Clemson Exp. Forest	12 Aug 2008
Culicoides villosipennis	A37	SC: Pickens Co. Clemson Exp. Forest	21 Aug 2007
Culicoides nanus	A42	SC. Richland Co. Congaree N.P.	14 May 2010
Culicoides stellifer	A66	SC. Richland Co. Congaree N.P.	7 Aug 2008
Culicoides debilipalpis	A67	SC. Richland Co. Congaree N.P.	14 May 2010
Culicoides nanus	A68	SC. Richland Co. Congaree N.P.	14 May 2010
Culicoides bickleyi	A69	SC. Richland Co. Congaree N.P.	5 May 2008
Culicoides spinosus	A70	SC. Richland Co. Congaree N.P.	5 May 2008
Culicoides spinosus	A71	SC. Richland Co. Congaree N.P.	5 May 2008
Culicoides hinmani	A72	SC. Richland Co. Congaree N.P.	12 Jun 2008

Table 2.1. *Continued*

Taxon	Code	Location	Date
Culicoides biguttatus	A73	SC. Richland Co. Congaree N.P.	12 Jun 2008
Culicoides haematopotus	A74	SC. Richland Co. Congaree N.P.	7 Aug 2008
Culicoides niger	A75	SC. Richland Co. Congaree N.P.	5 May 2008
Culicoides paraensis	A76	SC. Richland Co. Congaree N.P.	7 Aug 2008
Culicoides arboricola	A77	SC. Richland Co. Congaree N.P.	7 Aug 2008
Culicoides stellifer	A78	SC. Richland Co. Congaree N.P.	7 Aug 2008
Culicoides scanloni	A79	SC. Richland Co. Congaree N.P.	12 Jun 2008
Culicoides debilipalpis	A80	SC: McCormick Co. Hickory Knob S.P.	6 Aug 2009
Culicoides arboricola	A81	SC: McCormick Co. Hickory Knob S.P.	6 Aug 2009
Culicoides haematopotus	A83	SC. Richland Co. Congaree N.P.	7 Aug 2008
Culicoides scanloni	A84	SC. Richland Co. Congaree N.P.	12 Jun 2008
Culicoides furens	A87	SC: Charleston Co. Clemson CR&EC	10 Aug 2009
Culicoides bermudensis	AL1	AL: Mobile Co. Grand Bay Sav	19 Dec 2006
Culicoides mississippiensis	AL3	AL: Mobile Co. Brookley	20 Feb 2007
Culicoides villosipennis	BC5	SC: Barnwell Co. 33.37N, 81.41S	24 May 2007
Culicoides hollensis	CR6	SC: Charleston Co. Clemson CREC	18 Mar 2008
Culicoides hollensis	CR7	SC: Charleston Co. Clemson CREC	18 Mar 2008
Culicoides hollensis	CR15	SC: Charleston Co. Clemson CR&EC	18 Mar 2008
Culicoides haematopotus	CR29	SC: Charleston Co. Clemson CREC	10 Aug 2009

Table 2.1. *Continued.*

Taxon	Code	Location	Date
Culicoides guttipennis	EF1	SC: Pickens Co. Clemson Exp. Forest	22 May 2007
Culicoides spinosus	EF3	SC: Pickens Co. Clemson Exp. Forest	22 May 2007
Culicoides melleus	HB1	SC: Georgetown Co. Huntington Beach S.P.	29 Apr 2010
Culicoides furens	HB5	SC: Georgetown Co. Huntington Beach S.P.	29 Apr 2010
Culicoides tissoti	HB6	SC: Georgetown Co. Huntington Beach S.P.	29 Apr 2010
Culicoides debilipalpis	jul1	IL: Menard Co. Star Hill Arboretum	19 Jun 2009
Culicoides sonorensis	jul2	WY: Crook Co. Barlow Canyon	18 Jun 2008
Culicoides cockerellii	jul5	WY: Crook Co. Barlow Canyon	18 Jun 2008
Culicoides debilipalpis	jul6	IL: Menard Co. Star Hill Arboretum	19 Jun 2009
Culicoides doeringae	jul7	CO: Laramie Co. Horsetooth Reservoir	9 Jul 2008
Culicoides brookmani	jul8	WY: Crook Co. Barlow Canyon	18 Jun 2008
Culicoides travisi	jul9	SC: Charleston Co. Clemson CR&EC	4 May 2008
Culicoides palmerae	jul10	CO: Laramie Co. Horsetooth Reservoir	9 July 2008
Culicoides nanus	jul11	SC: Charleston Co. Clemson CR&EC	4 Mar 2008
Culicoides melleus	jul12	SC: Charleston Co. Clemson CR&EC	4 May 2008
Culicoides parapiliferus	WI5	WI: Juneau Co. Necedah Wildlife Refuge	2 Jun 2009
Culicoides haematopotus	WI6	WI: Juneau Co. Necedah Wildlife Refuge	9 Jun 2009
Culicoides bickleyi	WI10	WI: Juneau Co. Necedah Wildlife Refuge	13 Jun 2009
Culicoides stilobezzioides	WI12	WI: Juneau Co. Necedah Wildlife Refuge	8 Jun 2009

Table 2.1. *Continued.*

Taxon	Code	Location	Date
Culicoides crepuscularis	WI15	WI: Juneau Co. Necedah Wildlife Refuge	9 Jun 2009
Culicoides biguttatus	WI16	WI: Juneau Co. Necedah Wildlife Refuge	9 Jun 2009
Culicoides obsoletus	WI18	WI: Juneau Co. Necedah Wildlife Refuge	9 Jun 2009
Culicoides parapiliferus	WI19	WI: Juneau Co. Necedah Wildlife Refuge	1 Jun 2009
Culicoides sanguisuga	WI20	WI: Juneau Co. Necedah Wildlife Refuge	10 Jun 2009
Culicoides crepuscularis	WI21	WI: Juneau Co. Necedah Wildlife Refuge	22 Jun 2009
Culicoides obsoletus	WI23	WI: Juneau Co. Necedah Wildlife Refuge	8 Jun 2009
Culicoides obsoletus	WI24	WI: Juneau Co. Necedah Wildlife Refuge	8 Jun 2009
Culicoides venustus	(+)	SC: Barnwell Co. 33.37N, 81.41S	10 May 2007
Atrichopogon sp.	OG8	SC: Charleston Co. Clemson CR&EC	1 May 2010
Forcipomyia glauca	OG9	SC: Richmond Co. Congaree National Park	5 May 2008
Dasyhelea sp.	OG10	SC: Barnwell Co. 33.37N, 81.41S	14 Apr 2007
Ceratoculicoides virginianus	OG11	SC: Pickens Co. Clemson Exp. Forest	22 May 2007
Brachypogon canadensis	OG12	AL: Baldwin Co. Byrnes Lake	20 Apr 2007
Stilobezzia stonei	OG13	SC: Barnwell Co. 33.37N, 81.41S	10 May 2007
Alluaudomyia needhami	OG14	SC: Barnwell Co. 33.37N, 81.41S	10 May 2007
Monohelea floridensis	OG16	AL: Mobile Co. Camp Sid Edmunds	22 Aug 2006
Downeshelea stonei	OG17	SC: Barnwell Co. 33.37N, 81.41S	10 May 2007
Echinohelea lanei	OG18	SC: Barnwell Co. 33.37N, 81.41S	6 Jun 2007

Table 2.1. *Continued*.

Taxon	Code	Location	Date
Probezzia albitibia	OG19	SC: Pickens Co. Clemson Exp. Forest	5 Jun 2008
Bezzia nobilis	OG20	SC: Barnwell Co. 33.37N, 81.41S	14 Aug 2007

Table 2.2. Primers used to amplify a 523-bp fragment of COI from Ceratopogonidae.

Primer	**Sequence (5′-3′)**
C1-J-1718	GGAGGATTTGGAAATTGATTAGT
C1-N-2191	CAGGTAAAATTAAAATAAACTTCTGG

Table 2.3. Larvae identified, study code, site, BLAST search results, neighbor-joining cluster, and morphological identification. CR: Clemson Coastal Research and Education Center, HB: Huntington Beach State Park, WB: Woods Bay State Park, CP: Congaree National Park, HK: Hickory Knob State Resort Park, EF: Clemson Experimental Forest, TR: Table Rock State Park, JG: Jones Gap State Park.

Larval ID	**Site**	**Blast Sequence (% Identity)[1]**	**Neighbor-Joining Cluster[2]**	**Morphological Identification [3,4]**
L1	EF	*C. haematopotus* A1 (99)	*C. haematopotus*	?
L2	EF	*C. haematopotus* A1 (94)	*C. haematopotus*	?
L3	EF	*C. stellifer* A6 (86)	Species R	?
L4	EF	*C. stellifer* A6 (97)	Species T	?
L5	EF	*C. bickleyi* A69 (97)	*C. bickleyi* complex	*
L6	EF	*C. obsoletus* A7 (100)	*C. obsoletus*	?
L7	EF	*C. stellifer* A6 (87)	Species R	?
L8	EF	*C. haematopotus* A1 (94)	NI	—
L9	EF	*C. haematopotus* CR29 (95)	*C. haematopotus*	?
L10	EF	*C. stellifer* A6 (86)	Species R	?
L11	EF	*C. spinosus* A71 (98)	*C. spinosus*	?
L12	EF	*C. bickleyi* A69 (92)	NI	*
L13	EF	*C. spinosus* A71 (84)	Species V	?
L14	EF	*C. parapiliferus* WI19 (87)	*C. piliferus* group	—
L15	EF	*C. spinosus* A32 (99)	*C. spinosus*	?
L16	EF	*C. stellifer* A6 (84)	Species S	Species S

[1]—: Missing data, not positive amplification or specimen was not recovered from extraction
[2]NI: not included in the analysis because of sequence length or alignment issues
[3]?: Morphological identification could not be accurately assigned due to poor slide prep or artifacts in slide preparation
[4]*: Multiple morphospecies present, morphological identification could not be assigned.

Table 2.3. *Continued.*

L17	EF	*C. haematopotus* A1 (97)	*C. haematopotus*	—
L18	EF	*C. haematopotus* A74 (99)	*C. haematopotus*	—
L19	EF	*C. haematopotus* A74 (98)	*C. haematopotus*	?
L20	EF	*C. haematopotus* CR29 (98)	*C. haematopotus*	?
L21	EF	*C. haematopotus* A74 (98)	*C. haematopotus*	*C. haematopotus*
L22	EF	*C. bickleyi* A69 (88)	NI	?
L23	EF	*C. stellifer* A6 (96)	Species T	?
L24	EF	*C. spinosus* A32 (99)	*C. spinosus*	?
L25	EF	*C. spinosus* EF3 (97)	*C. spinosus*	—
L26	EF	*C. spinosus* A70 (98)	*C. spinosus*	—
L27	EF	*C. stellifer* A78 (99)	*C. stellifer*	?
L28	EF	*C. spinosus* EF3 (99)	*C. spinosus*	?
L29	EF	*C. stellifer* A6 (86)	Species S	Species S
L30	EF	—	—	?
L31	EF	*C. haematopotus* CR29 (98)	*C. haematopotus*	—
L32	EF	*C. haematopotus* A74 (98)	*C. haematopotus*	?
L33	EF	*C. stellifer* A6 (84)	Species S	Species S
L34	EF	*C. stellifer* A6 (86)	Species O	Species O
L35	EF	*C. stellifer* A6 (85)	Species S	Species S
L36	EF	*C. bickleyi* A69 (96)	*C. bickleyi* complex	*
L37	EF	*C. stellifer* A78 (97)	*C. stellifer*	*C. stellifer*
L38	EF	*C. haematopotus* CR29 (99)	*C. haematopotus*	?
L39	EF	*C. haematopotus* A1 (100)	*C. haematopotus*	—
L40	EF	*C. stellifer* A78 (95)	*C. stellifer*	*C. stellifer*
L41	EF	*C. stellifer* A6 (87)	Species R	?
L42	EF	*C. haematopotus* A74 (98)	*C. haematopotus*	*C. haematopotus*

Table 2.3. *Continued.*

L43	EF	*C. stellifer* A6 (84)	Species S	—
L44	EF	*C. sonorensis* jul-2 (87)	Species D	Species D
L45	EF	*C. bickleyi* A69 (96)	*C. bickleyi* complex	—
L46	EF	*C. stellifer* A6 (84)	Species S	?
L47	EF	*C. bickleyi* A69 (100)	*C. bickleyi* complex	—
L48	EF	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L49	EF	*C. haematopotus* WI6 (97)	*C. haematopotus*	—
L50	EF	*C. haematopotus* CR29 (95)	*C. haematopotus*	?
L51	EF	—	—	?
L52	EF	*C. spinosus* A70 (99)	*C. spinosus*	?
L53	EF	*C. haematopotus* CR29 (98)	*C. haematopotus*	*C. haematopotus*
L54	EF	*C. spinosus* EF3 (97)	*C. spinosus*	?
L55	EF	*C. haematopotus* A74 (98)	*C. haematopotus*	?
L56	EF	*C. spinosus* A70 (94)	*C. spinosus*	?
L57	EF	*C. stellifer* A78 (98)	*C. stellifer*	?
L58	EF	*C. stellifer* A6 (85)	Species S	?
L59	EF	*C. spinosus* A70 (97)	*C. spinosus*	?
L60	EF	*C. stellifer* A6 (84)	Species S	Species S
L61	EF	*C. stellifer* A6 (84)	Species S	Species S
L62	EF	*C. stellifer* A6 (84)	Species S	?
L63	EF	*C. spinosus* A9 (98)	*C. spinosus*	?
L64	EF	—	—	?
L65	EF	*C. stellifer* A6 (87)	Species R	Species R
L66	EF	*C. haematopotus* A74 (98)	NI	—
L67	EF	*C. stellifer* A6 (98)	Species T	? (Not species T)
L68	EF	*C. haematopotus* A74 (92)	NI	—

Table 2.3. *Continued.*

L69	EF	—	—	—
L70	HK	*C. haematopotus* A1 (99)	*C. haematopotus*	?
L71	HK	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L72	HK	*C. haematopotus* CR29 (98)	*C. haematopotus*	*C. haematopotus*
L73	HK	—	—	?
L74	HK	*C. haematopotus* CR29 (99)	*C. haematopotus*	*C. haematopotus*
L75	HK	*C. biguttatus* A10 (98)	*C. biguttatus*	?
L76	HK	*C. haematopotus* CR29 (98)	*C. haematopotus*	?
L77	CP	*C. furens* HB5 (88)	Species A	Species A
L78	CP	*C. haematopotus* A1 (97)	*C. haematopotus*	*C. haematopotus*
L79	CP	*C. stellifer* A6 (98)	Species T	Species T
L80	CP	*C. furens* HB5 (88)	Species A	Species A
L81	CP	*C. stellifer* A6 (98)	Species T	Species T
L82	CP	*C. stellifer* A6 (97)	Species T	Species T
L83	CP	*C. bickleyi* A69 (97)	*C. bickleyi* complex	*
L84	CP	*C. stellifer* A6 (98)	Species T	Species T
L85	CP	—	—	*C. haematopotus*
L86	CP	*C. parapiliferus* WI19 (91)	*C. piliferus* group	*C. piliferus* group
L87	CP	*C. scanloni* A79 (98)	*C. piliferus* group	*C. piliferus group*
L88	CP	*C. parapiliferus* WI5 (98)	*C. parapiliferus*	*C. parapiliferus*
L89	CP	*C. spinosus* EF3 (86)	Species M	Species M
L90	CP	—	—	*C. parapiliferus*
L91	CP	*C. bickleyi* A69 (97)	*C. bickleyi* complex	*
L92	CP	*C. bickleyi* A69 (98)	*C. bickleyi* complex	*
L93	CP	*C. stellifer* A6 (98)	Species T	Species T
L94	CP	*C. furens* HB5 (85)	Species P	Species P

Table 2.3. *Continued.*

L95	CP	*C. stellifer* A78 (98)	*C. stellifer*	*C. stellifer*
L96	CP	*C. stellifer* A6 (98)	Species T	Species T
L97	CP	*C. stellifer* A6 (98)	Species T	Species T
L98	CP	*C. stellifer* A6 (98)	Species T	?
L99	CP	*C. stellifer* A6 (99)	Species T	?
L100	CP	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L101	CP	*C. haematopotus* A1 (97)	*C. haematopotus*	*C. haematopotus*
L102	CP	*C. bickleyi* A69 (96)	*C. bickleyi* complex	*
L103	CP	*C. biguttatus* A10 (94)	NI	(*C. biguttatus*)
L104	CP	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L105	CP	*C. stellifer* A6 (98)	Species T	Species T
L106	CP	*C. bickleyi* A69 (97)	*C. bickleyi* complex	*
L107	CP	*C. stellifer* A6 (98)	Species T	Species T
L108	CP	*C. stellifer* A6 (97)	Species T	Species T
L109	CP	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L110	CP	*C. parapiliferus* WI5 (99)	*C. parapiliferus*	*C. parapiliferus*
L111	CP	*C. parapiliferus* WI5 (99)	*C. parapiliferus*	*C. parapiliferus*
L112	CP	*C. stellifer* A6 (98)	Species T	Species T
L113	CP	*C. stellifer* A6 (98)	Species T	Species T
L114	CP	*C. haematopotus* A1 (95)	*C. haematopotus*	*C. haematopotus*
L115	CP	—	—	Species T
L116	CP	*C. stellifer* A6 (99)	Species T	Species T
L117	CP	*C. bickleyi* A69 (97)	*C. bickleyi* complex	*
L118	CP	*C. haematopotus* A1 (96)	*C. haematopotus*	*C. haematopotus*
L119	CP	*C. bickleyi* A69 (95)	*C. bickleyi* complex	*
L120	CP	*C. bickleyi* A69 (95)	*C. bickleyi* complex	*

Table 2.3. *Continued.*

L121	CP	*C. parapiliferus* WI5 (99)	*C. parapiliferus*	*C. parapiliferus*
L122	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L123	CR	*C. hollensis* CR7 (98)	*C. hollensis*	*C. hollensis*
L124	CR	*C. furens* HB5 (99)	*C. furens*	?
L125	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L126	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L127	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L128	CR	*C. spinosus* A71 (87)	Species G	?
L129	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L130	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L131	CR	*C. hollensis* CR6 (99)	*C. hollensis*	*C. hollensis*
L132	CR	*C. hollensis* CR7 (93)	*C. hollensis*	?
L133	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L134	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L135	CR	*C. spinosus* A71 (87)	Species I	Species I
L136	CR	*C. haematopotus* CR29 (99)	*C. haematopotus*	?
L137	CR	—	—	Species I
L138	CR	*C. furens* HB5(99)	*C. furens*	*C. furens*
L139	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L140	CR	*C. furens* HB5 (99)	*C. furens*	?
L141	CR	C. venustus A81 (82)	Species Q	?
L142	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L143	CR	*C. hollensis* CR6 (99)	*C. hollensis*	*C. hollensis*
L144	CR	*C. sonorensis* jul2 (87)	Species D	Species D
L145	CR	*C. furens* HB5 (99)	*C. furens*	?
L146	CR	*C. haematopotus* CR29 (100)	*C. haematopotus*	*C. haematopotus*

Table 2.3. *Continued.*

L147	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L148	CR	*C. hollensis* CR7 (98)	*C. hollensis*	*C. hollensis*
L149	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L150	CR	*C. spinosus* A70 (86)	Species H	Species H
L151	CR	*C. haematopotus* CR29 (99)	*C. haematopotus*	?
L152	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L153	CR	*C. spinosus* A71 (87)	Species G	Species G
L154	CR	*C. spinosus* A71 (87)	Species I	Species I
L155	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L156	CR	*C. hollensis* CR7 (98)	*C. hollensis*	*C. hollensis*
L157	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L158	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L159	CR	*C. hollensis* CR6 (97)	*C. hollensis*	*C. hollensis*
L160	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L161	CR	*C. spinosus* A70 (86)	Species H	?
L162	CR	*C. spinosus* A71 (87)	Species I	Species I
L163	CR	*C. haematopotus* A1 (97)	*C. haematopotus*	*C. haematopotus*
L164	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L165	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L166	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L167	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L168	CR	*C. hollensis* CR7 (98)	*C. hollensis*	*C. hollensis*
L169	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L170	CR	*C. hollensis* CR7 (98)	*C. hollensis*	*C. hollensis*
L171	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L172	CR	*C. haematopotus* A1 (97)	*C. haematopotus*	*C. haematopotus*

Table 2.3. *Continued.*

L173	CR	*C. spinosus* A71 (87)	Species I	Species I
L174	CR	*C. sonorensis* jul2 (87)	Species D	Species D
L175	CR	*C. melleus* HB1 (87)	Species G	Species G
L176	CR	*C. spinosus* A71 (87)	Species G	?
L177	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L178	CR	*C. haematopotus* A1 (97)	*C. haematopotus*	*C. haematopotus*
L179	CR	—	—	*C. hollensis*
L180	CR	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L181	CR	*C. crepuscularis* WI15 (87)	Species L	Species L
L182	CR	*C. bickleyi* A69 (96)	*C. bickleyi* complex	*
L183	CR	*C. hollensis* CR7 (99)	*C. hollensis*	*C. hollensis*
L184	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L185	CR	*C. melleus* HB1 (98)	*C. melleus*	? (not *melleus*)
L186	CR	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L187	CR	*C. bickleyi* A69 (88)	Species B	?
L188	CR	—	—	?
L189	TR	*C. haematopotus* CR29 (98)	*C. haematopotus*	*C. haematopotus*
L190	TR	*C. stellifer* A6 (85)	Species S	?
L191	TR	*C. stellifer* A6 (85)	Species S	Species S
L192	TR	—	—	*C. haematopotus*
L193	TR	*C. haematopotus* CR29 (95)	*C. haematopotus*	?
L194	TR	*C. stellifer* A6 (84)	Species S	Species S
L195	TR	—	—	?
L196	TR	*C. haematopotus* A1 (99)	*C. haematopotus*	*C. haematopotus*
L197	TR	*C. haematopotus* A1 (99)	*C. haematopotus*	*C. haematopotus*
L198	TR	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*

Table 2.3. *Continued.*

L199	TR	*C. sonorensis* jul2 (87)	Species D	Species D
L200	TR	—	—	Species T
L201	TR	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L202	TR	*C. haematopotus* A1 (99)	*C. haematopotus*	*C. haematopotus*
L203	TR	—	—	?
L204	TR	—	—	—
L205	TR	*C. obsoletus* WI20 (86)	Species R	?
L206	TR	—	—	Not *Culicoides*
L207	TR	—	—	?
L208	TR	—	—	?
L209	TR	—	—	?
L210	WB	—	—	?
L211	WB	*C. niger* A75 (97)	*C. niger*	*C. niger*
L212	WB	—	—	Not *Culicoides*
L213	WB	*C. sonorensis* jul2 (88)	Species D	Species D
L214	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L215	WB	*C. furens* HB5 (85)	Species E	Species E
L216	WB	*C. niger* A75 (86)	Species K	Species K
L217	WB	*C. furens* A87 (86)	Species J	Species J
L218	WB	*C. spinosus* A70 (86)	Species N	?
L219	WB	*C. parapiliferus* WI5 (98)	*C. parapiliferus*	?
L220	WB	*C. crepuscularis* WI15 (86)	Species K	Species K
L221	WB	*C. furens* HB5 (99)	*C. furens*	Not *C. furens*
L222	WB	—	—	Not *Culicoides*
L223	WB	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L224	WB	*C. spinosus* A70 (86)	Species N	Species N

Table 2.3. *Continued.*

L225	WB	*C. haematopotus* CR29 (98)	*C. haematopotus*	*C. haematopotus*
L226	WB	*C. stellifer* A6 (96)	Species T	Species T
L227	WB	*C. niger* A75 (86)	Species K	Species K
L228	WB	*C. parapiliferus* WI5 (98)	*C. parapiliferus*	*C. parapiliferus*
L229	WB	*C. niger* A75 (97)	*C. niger*	*C. niger*
L230	WB	*C. bickleyi* A69 (97)	Species C	*
L231	WB	*Echinohelea lanei* (98)	Species U	Not *Culicoides*
L232	WB	*C. parapiliferus* WI5 (98)	*C. parapiliferus*	?
L233	WB	*C. niger* A75 (97)	*C. niger*	*C. niger*
L234	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L235	WB	*C. furens* HB5 (97)	*C. furens*	Species T
L236	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L237	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L238	WB	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L239	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L240	WB	*C. bickleyi* A69 (94)	*C. bickleyi* complex	*
L241	WB	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L242	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L243	WB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L244	HB	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L245	HB	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L246	HB	*C. bickleyi* A69 (99)	*C. bickleyi* complex	*
L247	HB	*C. furens* HB5 (99)	*C. furens*	?
L248	HB	*C. furens* HB5 (99)	*C. furens*	?
L249	HB	*C. bickleyi* A69 (100)	*C. bickleyi* complex	*
L250	HB	*C. furens* HB5 (98)	*C. furens*	*C. furens*

Table 2.3. *Continued.*

L251	HB	*C. furens* HB5 (94)	*C. furens*	*C. furens*
L252	HB	*C. bickleyi* A69 (98)	*C. bickleyi* complex	*
L253	HB	*C. melleus* HB1 (99)	*C. melleus*	*C. melleus*
L254	HB	*C. melleus* HB1 (99)	*C. melleus*	*C. melleus*
L255	HB	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L256	HB	*C. furens* HB5 (98)	*C. furens*	?
L257	HB	*C. furens* HB5 (96)	*C. furens*	?
L258	HB	*C. melleus* HB1 (100)	*C. melleus*	*C. melleus*
L259	HB	*C. melleus* HB1 (100)	*C. melleus*	*C. melleus*
L260	HB	No hits	NI	?
L261	HB	—	—	*C. hollensis*
L262	HB	*C. furens* HB5 (98)	NI	*C. furens*
L263	HB	*C. bickleyi* A69 (96)	Species C	?
L264	HB	*C. melleus* HB1 (99)	*C. melleus*	*C. melleus*
L265	HB	*C. furens* HB5 (98)	*C. furens*	?
L266	HB	—	—	*C. melleus*
L267	HB	*C. bickleyi* A69 (92)	Species F	Species F
L268	HB	*C. furens* HB5 (97)	*C. furens*	*C. furens*
L269	HB	*C. furens* HB5 (95)	*C. furens*	?
L270	HB	*C. melleus* HB1 (99)	*C. melleus*	*C. melleus*
L271	HB	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L272	HB	*C. hollensis* CR6 (93)	*C. hollensis*	*C. hollensis*
L273	HB	—	—	Not *Culicoides*
L274	HB	*C. furens* A87 (94)	*C. furens*	?
L275	HB	*C. melleus* HB1 (99)	*C. melleus*	*C. melleus*
L276	HB	—	—	*C. hollensis*

Table 2.3. *Continued.*

L277	HB	*C. melleus* HB1 (99)	*C. melleus*	*C. melleus*
L278	HB	*C. furens* HB5 (98)	*C. furens*	*C. furens*
L279	HB	—	—	*C. hollensis*
L280	HB	*C. furens* A87 (98)	*C. furens*	?
L281	HB	*C. furens* A87 (99)	*C. furens*	?
L282	HB	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L283	HB	*C. furens* HB5 (99)	*C. furens*	*C. furens*
L284	HB	*C. furens* HB5 (99)	*C. furens*	?
L285	HB	*C. furens* HB5 (97)	*C. furens*	?
L286	HB	*C. furens* A87 (97)	*C. furens*	?
L287	HB	—	—	*C. hollensis*
L288	HB	*C. furens* HB5 (99)	*C. furens*	—
L289	JG	—	—	Not *Culicoides*
L290	JG	*C. haematopotus* A74 (99)	*C. haematopotus*	*C. haematopotus*
L291	JG	—	—	?
L292	JG	*C. haematopotus* A74 (98)	*C. haematopotus*	*C. haematopotus*
L293	JG	*C. haematopotus* A1 (98)	*C. haematopotus*	*C. haematopotus*
L294	JG	—	—	?
L295	JG	—	—	?
L296	JG	—	—	*C. haematopotus*
L297	JG	—	—	?
L298	JG	—	—	?
L299	JG	—	—	*C. haematopotus*
L300	JG	—	—	*C. haematopotus*
L301	JG	—	—	*C. haematopotus*
L302	JG	—	—	?

Table 2.3. *Continued.*

L303	JG	—	—	*C. haematopotus*
L304	JG	*C. haematopotus* A74 (97)	*C. haematopotus*	*C. haematopotus*
L305	JG	—	—	Not *Culicoides*
L306	JG	—	—	?
L307	JG	—	—	?
L308	JG	—	—	?
L309	JG	—	—	Not *Culicoides*
L310	JG	—	—	Not Culicoides
L311	JG	—	—	?
L312	JG	—	—	Not *Culicoides*
L313	JG	*C. haematopotus* A74 (98)	*C. haematopotus*	—
L314	JG	—	—	?
L315	JG	—	—	?
L316	JG	—	—	?
L317	JG	—	—	?
L318	JG	—	—	Not *Culicoides*
L319	JG	—	—	Not *Culicoides*
L320	JG	—	—	Not *Culicoides*
L321	JG	—	—	?
L322	JG	—	—	Not *Culicoides*
L323	JG	—	—	Not *Culicoides*

Table 2.4. Percentage of substrate samples by site with larval *Culicoides*. Numbers of samples are in parentheses. CR = Clemson Coastal Research and Education Center, HB = Huntington Beach State Park, WB = Woods Bay State Park, CP = Congaree National Park, HK = Hickory Knob State Resort Park, EF = Clemson Experimental Forest, TR = Table Rock State Park, JG = Jones Gap State Park, bic = *Culicoides bickleyi* complex, big = *C. biguttatus*, fur = *C. furens*, hae = *C. haematopotus*, hol = *C. hollensis*, mel = *C. melleus*, obs = *C. obsoletus*, nig = *C. niger*, par = *C. parapiliferus*, pil = *C. piliferus* group, spi = *C. spinosus*, ste = *C. stellifer*, R = *Culicoides* sp. R, S = *Culicoides* sp. S, T = *Culicoides* sp. T.

Site/ Taxon	**bic**	**big**	**fur**	**hae**	**hol**	**mel**	**obs**	**nig**	**par**	**pil**	**spi**	**ste**	**R**	**S**	**T**	**Site Total**
CR (79)	1.3	0.0	30.4	7.6	21.5	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	60.8
HB (51)	5.9	0.0	45.1	0.0	9.8	17.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	76.5
WB (82)	13.4	0.0	0.0	2.4	0.0	0.0	0.0	3.7	3.7	0.0	0.0	0.0	0.0	0.0	2.4	20.7
CP (80)	13.8	1.3	0.0	5.0	0.0	0.0	0.0	0.0	6.3	2.5	0.0	1.3	0.0	0.0	16.3	31.3
HK (45)	0.0	2.2	2.2	12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.3
EF (96)	5.2	0.0	0.0	12.5	0.0	0.0	1.0	0.0	0.0	1.0	7.3	4.2	5.2	9.4	3.1	32.3
TR (48)	4.2	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	6.3	2.1	25.0
JG (56)	0.0	0.0	0.0	17.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	17.9
All Sites (537)	6.1	0.4	8.9	8.2	4.1	1.8	0.2	0.6	1.5	0.6	1.3	0.9	1.1	2.2	3.5	35.0

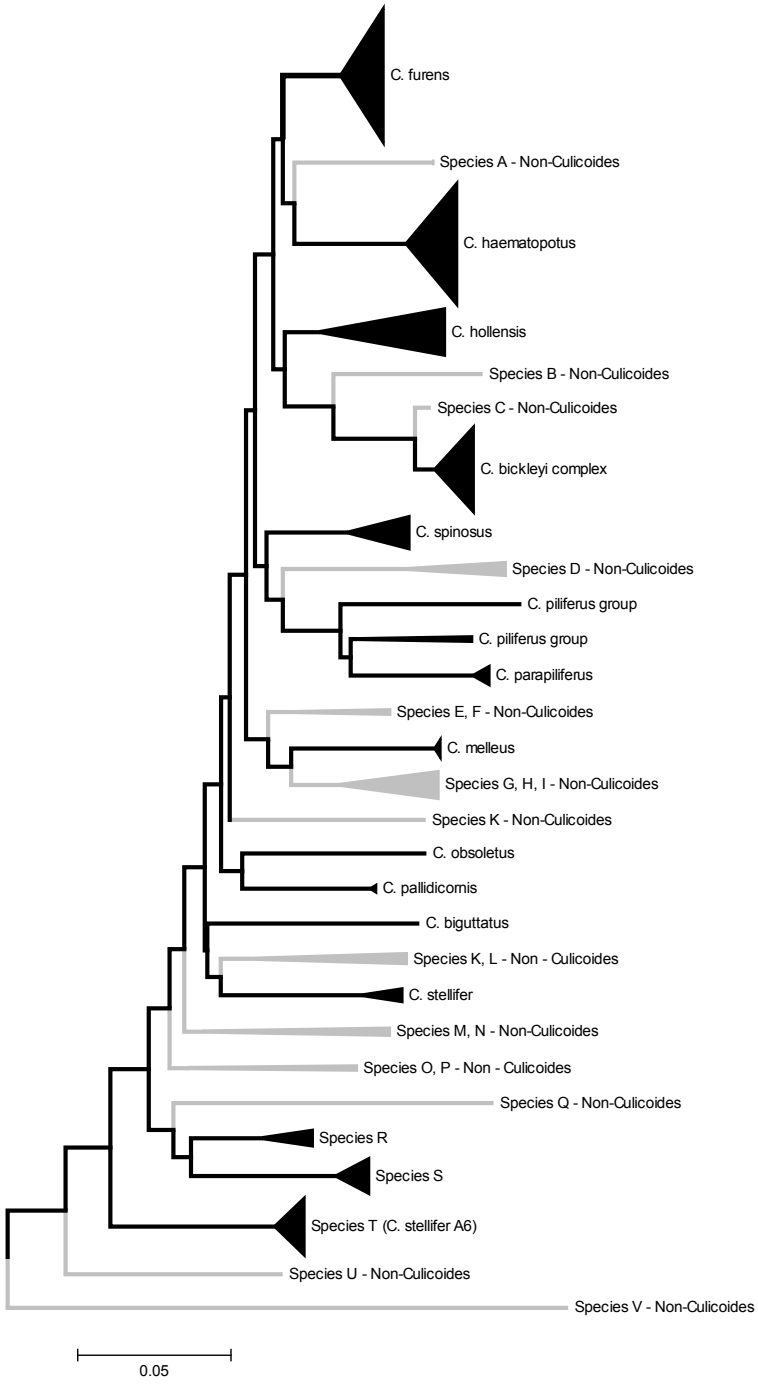


Figure 2.4. Compressed neighbor-joining tree based on partial COI sequences of larvae of *Culicoides*. Black branches represent confirmed or probable *Culicoides* species; gray branches represent confirmed non-*Culicoides* species.

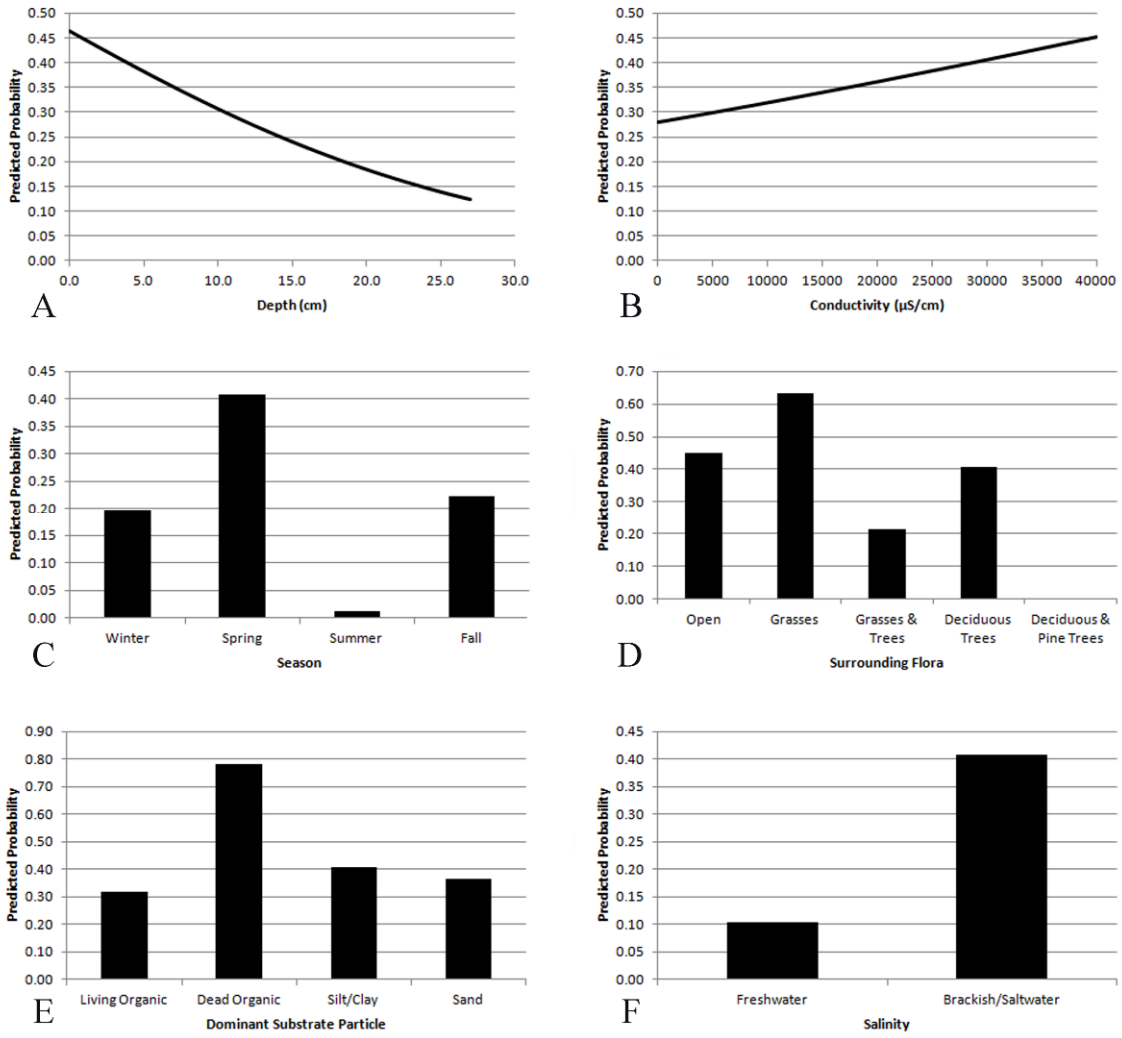


Figure 2.5. Probability plots for presence of *Culicoides furens* for variables retained in multiple logistic regression after stepwise Akaike Information Criterion (AIC) selection.

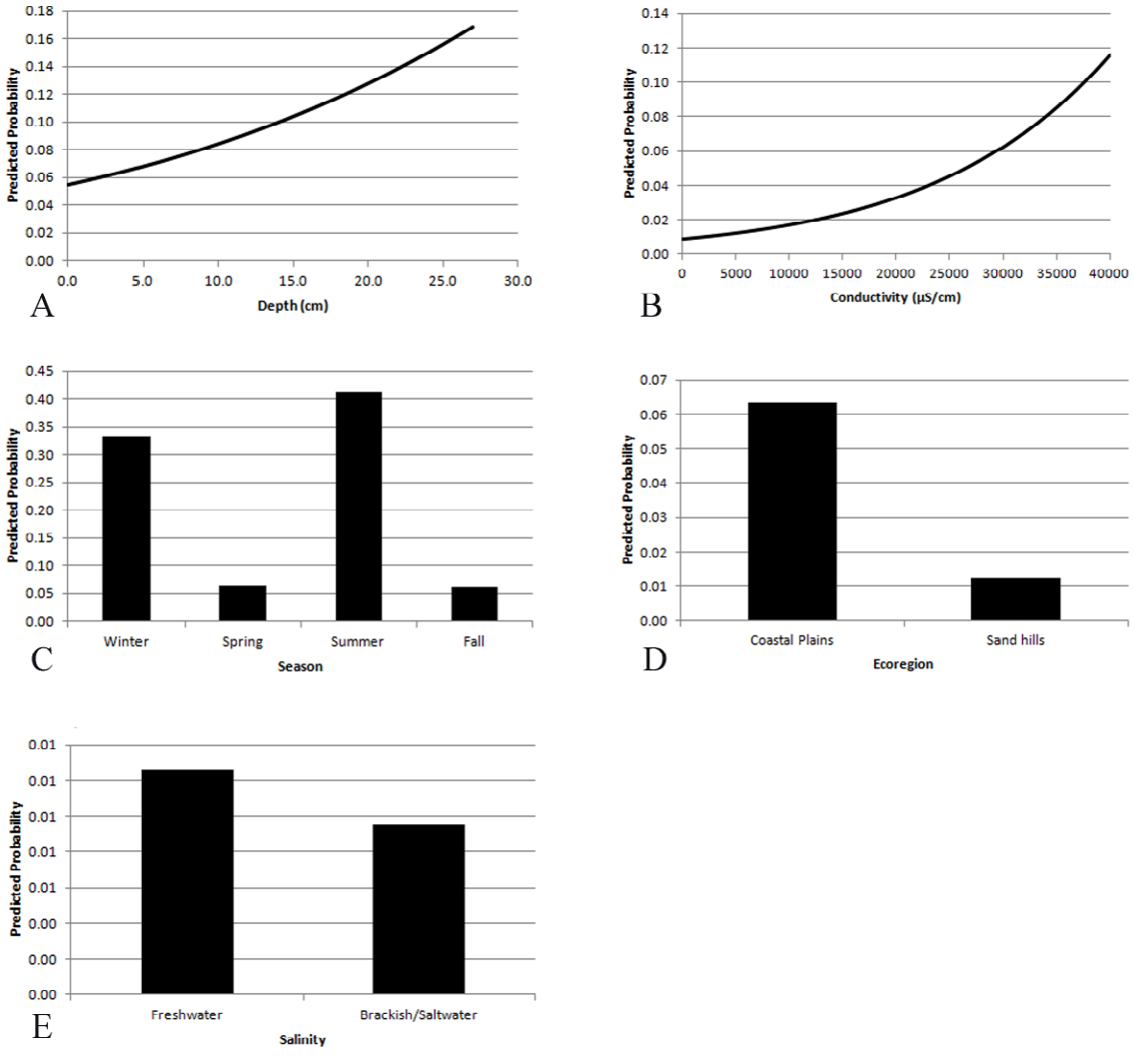


Figure 2.6. Probability plots for presence of *Culicoides hollensis* for variables retained in multiple logistic regression after stepwise Akaike Information Criterion (AIC) selection.

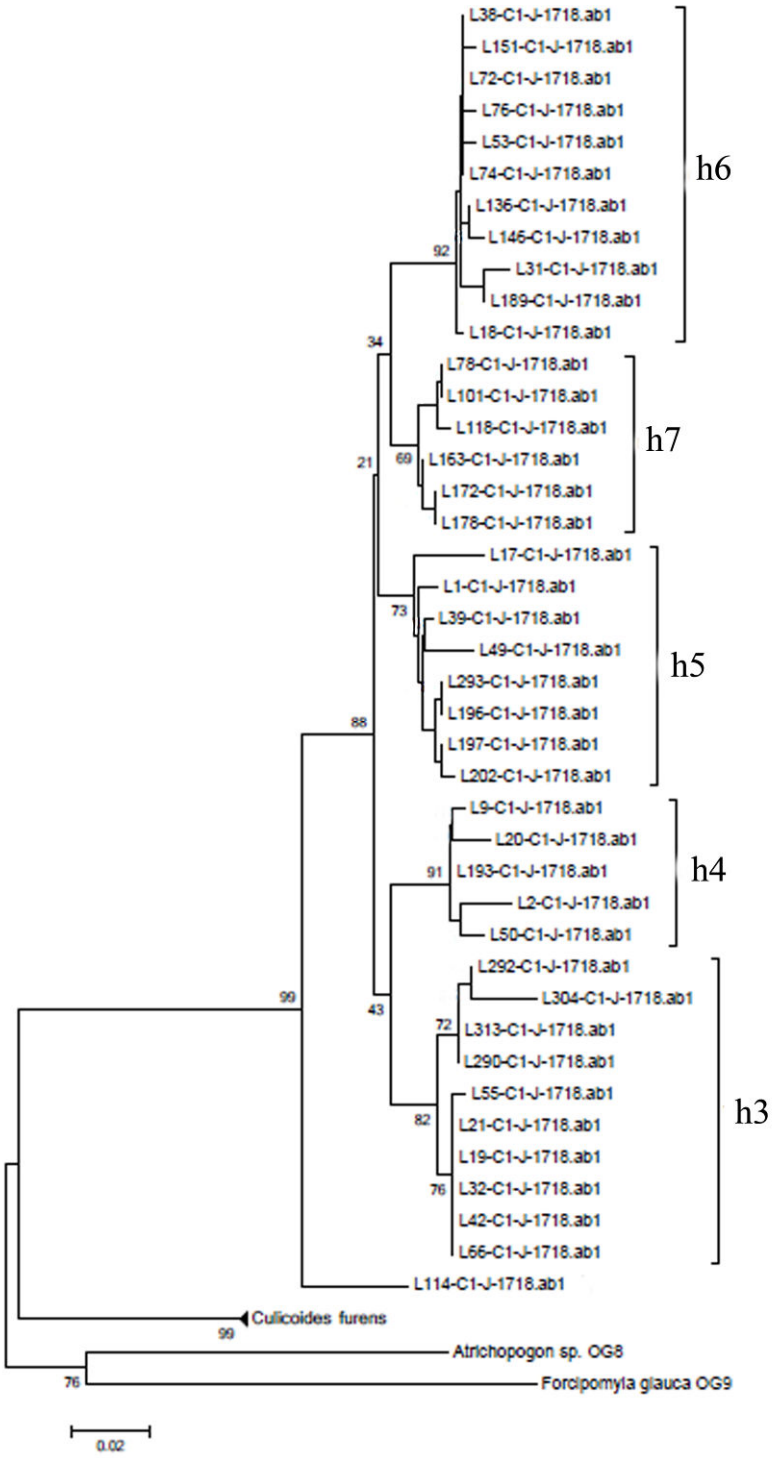


Figure 2.7. Maximum likelihood tree based on COI sequences from larval *Culicoides haematopotus*. Clade designations on right indicate groups used in discriminant analysis.

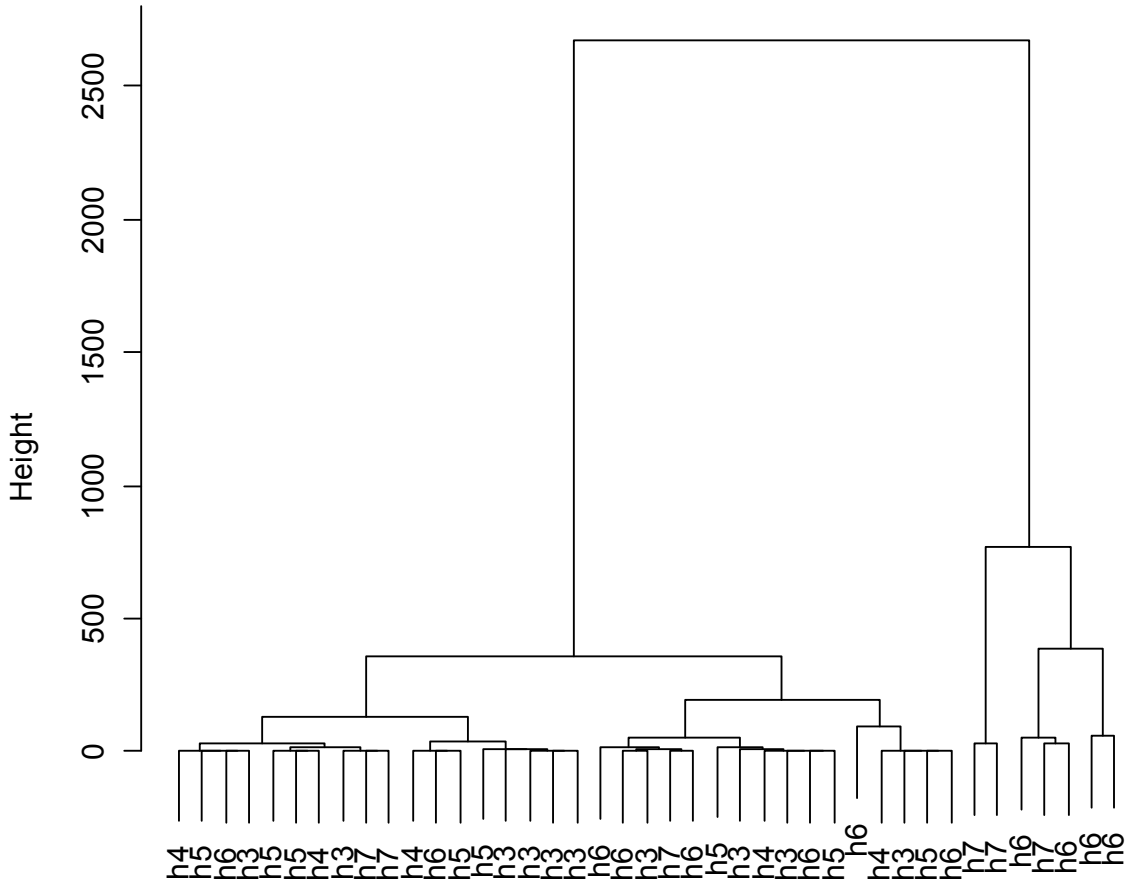


Figure 2.8. Cluster dendrogram resulting from hierarchical cluster analysis (Ward clustering method) of ecological data of five lineages of larval *C. haematopotus.* Codes (e.g., h4, h5) represent the five neighbor-joining clusters.

CHAPTER THREE

FUNCTIONAL MORPHOLOGY AND PHYLOGENETIC VALUE OF A SCUTAL STRUCTURE UNIQUE TO THE CERATOPOGONIDAE

Introduction

The family Ceratopogonidae is a well-supported monophyletic group, with as many as 10 synapomorphies reported for the family (Borkent and Craig 2004). The monophyly of the four extant subfamilies Leptoconopinae, Forcipomyiinae, Dasyheleinae, and Ceratopogoninae also are well supported (Borkent 1995, Borkent 2000, Borkent and Craig 2004). The relationships of the genera within the Leptoconopinae, Forcipomyiinae, and Dasyheleinae are fairly well resolved, whereas those among the genera of the subfamily Ceratopogoninae are not well resolved (Borkent 1995, Borkent 2000, Borkent and Craig 2004).

The subfamily Ceratopogoninae is organized into 6 tribes and 119 genera (Borkent 2012). The tribes Heteromyiini (8 genera), Sphaeromiini (28 genera), Palpomyiini (6 genera), and Stenoxini (2 genera) form a monophyletic group, though monophyly of each tribe is questionable (Borkent 1995, Borkent and Craig 2004). The tribe Ceratopogonini is the largest and is likely a paraphyletic group (Borkent 1995, Borkent 2000). The tribe Culicoidini (3 genera) is sister to the other tribes of Ceratopogoninae (Borkent 1995, Borkent and Craig 2004). No strong synapomorphies have been reported for the Culicoidini even though *Culicoides,* the largest and most economically important genus in the family, is in this tribe. Borkent (1995) reported one potential synapomorphy for the *Culicoides* or the *Culicoides*+*Paradasyhelea*, the

presence of sensilla coeloconica beyond flagellomere 1, but this character also occurs in other genera in the tribe Ceratopogonini (Borkent 1995).

Upon examining specimens of *Culicoides*, a character on the scutum was investigated as a potential synapomorphy within the genus and the family. This character consisted of two smooth areas of cuticle just anterior to the scutellum. These structures have been mentioned previously in the literature, but the function has not been demonstrated. Tokunaga (1937) referred to the structures as pore-like depressions, Wirth and Blanton (1959) referred to them as sensory areas, and Wirth and Hubert (1989) called them caudoscutellar pits. The structures are visible in scanning electron micrographs of Zaman (1983), but no close-up images were provided. None of these authors suggested a function for these structures, or at least a function supported by empirical evidence. Tokunaga (1937) briefly discussed the taxonomic value of these scutal structures, but a more thorough study is needed. For the purpose of this study, these smooth, small areas of the scutum will be referred to as scutal areolae, derived from the Latin terms *scutum,* meaning a shield, and *areolae,* meaning little open areas. This term does not invoke function and only describes the morphology of the structures.

The objectives of this study were to assess the function of the scutal structures and their phylogenetic value within the Ceratopogonidae. Four hypotheses were tested as to the function of the scutal areolae: 1) secretion of pheromones, 2) muscular attachment, 3) sensory, and 4) reflectance.

Materials and Methods

Survey of Scutal Areolae within the Ceratopogonidae

Various species of Ceratopogonidae in multiple genera were examined for the presence of scutal areolae (Table 3.1). Specimens included ethanol and slide mounted specimens observed by means of stereo- and compound microscopy.

Specimens of *Leptoconops americanus* Carter, 1921*, Austroconops mcmillani* Wirth and Lee, 1958*, Culicoides hollensis, Culicoides furens, Stilobezzia thomsenae* Wirth, 1953*,* and *Bezzia nobilis* (Winnertz), 1852, were prepared for further examination by scanning electron microscopy (SEM). Specimens were dehydrated by transitioning into 100% ethanol and drying in hexamethyldisilazane (HMDS). Specimens were mounted on carbon-graphite tape, affixed to aluminum stubs, sputter coated with platinum for 60-90 s, and imaged by means of a Hitachi TM3000 (analytical) at variable pressure.

Pupae and pupal exuviae of *Culicoides* and *Stilobezzia* were examined to assess the presence of scutal areolae. Pharate pupae of *C. guttipennis* were dehydrated and imaged with SEM as described above. Pupal exuviae of slide-mounted *C. denticulatus* and *S. bulla* were examined with compound light microscopy for scutal areolae.

Functional Morphology

To test the secretion hypothesis, scutal areolae were examined for the presence of pores. Specimens of *C. hollensis* were dehydrated as previously described and examined with variable pressure SEM without sputter coating. This method excluded the possibility that pores were concealed by platinum during sputter coating. Additional specimens were

cleared in lactic acid and the scutum and scutellum dissected from the thorax. The specimens were then dehydrated as previously described and examined internally for the presence of pores by variable pressure SEM, using sputter coated and non-sputter coated specimens.

To test the muscular attachment hypothesis, specimens of *C. hollensis* were cleared in lactic acid and the scutum and scutellum dissected from the thorax. The specimens were dehydrated as previously described and the scutal areolae examined internally by means of variable pressure SEM to assess the presence of internal apodemes. Additional specimens of *Culicoides* were collected by means of carbon dioxide-baited ultraviolet Centers for Disease Control and Prevention (CDC) traps from the Clemson University Experimental Forest for dissection. Specimens were transported to the laboratory and placed at -20°C for 5 min. Midges were placed in physiological saline, the scutum and scutellum were removed from the thorax, and the scutal areolae were examined internally for muscle attachment, using compound microscopy.

The possibility of a sensory function was tested by examining the scutal areolae for innervation. *Culicoides* collected from the experimental forest were placed in physiological saline and the scutum and scutellum dissected from the thorax. The tissue of interest was stained with 0.025% methylene blue for 10 min and destained in distilled water. The presence of nerve tissue associated with the scutal areolae was assessed by means of compound light microscopy.

The hypothesis of the scutal areolae serving as reflective structures was tested by using freshly collected *Culicoides* and ethanol-fixed *A. mcmillani*. Specimens of

Culicoides were collected as previously described and examined by means of stereomicroscopy. The reflectance properties of the scutal areolae were assessed visually by using stereomicroscopy and shining light on the structures at various angles and intensities.

Phylogenetic Analysis

The phylogenetic relationships among the extant taxa (genera and tribes) of Ceratopogonidae were analyzed using maximum parsimony. The family Chironomidae was used as the outgroup for the phylogenetic analysis (Borkent and McKeever 1990, Sinclair 1992, Wood and Borkent 1989). Characters from previous phylogenetic studies and two new characters were used to resolve relationships among taxa (Table 3.2). These characters included characters 1-32 and 34-57 of Borkent and Craig (2004) and characters 28-46, 48-52, and 54 of Borkent (1995). Character 33 of Borkent and Craig (2004) was not used because it was an autapomorphy for the extinct genus *Fossileptoconops*. Characters 1-26 of Borkent (1995) were not used because they were repeats of Borkent and Craig (2004) or were shown not to be synapomorphies in subsequent studies (Borkent 2000). Characters 27, 47, and 53 of Borkent (1995) were omitted because of poor support and homoplasy associated with these characters. Character 28 of Borkent (1995) (plesiomorphic: palisade setae absent on 1st hind tarsomere, apomorphic': partial row of palisade setae present on 1st hind tarsomere, apomorphic": complete row of palisade setae present on 1st hind tarsomere) was reduced to a two-state character (plesiomorphic: palisade setae absent, apomorphic: palisade setae present) because the polarization of this character is ambiguous. The intermediate state of

a partial row of setae could be a partial loss of setae just as likely as an initial gain. In addition to these characters, two new characters based on the morphology of scutal areolae were added (Table 3.2).

A phylogeny for the genera and tribes was reconstructed by using PAUP* 4.0b10 (Sinauer Associates, Inc., Sunderland, MA). A heuristic search by means of tree bisection with reconnection under maximum parsimony criterion was performed. The subsequent tree was saved and printed in Treeview (Page 1996).

Results

General Morphology

Scutal areolae were observed in the genera *Austroconops, Ceratopogon, Culicoides, Fanthamia, Leptoconops, Macrurohelea, Paradasyhelea, Stilobezzia,* and *Washingtonhelea* but not observed in the Forcipomyiinae, Dasyheleinae, and a majority of the Ceratopogoninae (Table 3.1). The scutal areolae were composed of smooth cuticle, lacking microtrichia and pores (Fig. 3.1). In *Austroconops, Leptoconops,* and *Stilobezzia,* the scutal areolae were relatively flush with the cuticle of the scutum (Fig. 3.2). In *Culicoides*, the scutal areolae were noticeably raised from the cuticle (Fig. 3.1). This raised condition was noticeable in *Paradasyhelea* without SEM when viewed laterally. No differences in scutal areolae were observed between male and female *C. hollensis* (Fig. 3.1) or *S. thomsenae*.

Scutal areolae were not observed in pupae but the corresponding areas could be observed in pharate pupae and pupal exuviae. Scanning electron micrographs of pharate pupae of *C. guttipennis* showed small indentions and wrinkling in the areas

corresponding to scutal areolae in the adult (Fig. 3.3A, B). No pores or openings were associated with these areas in pupae (Fig. 3.3B). Examination of pupal exuviae of *C. denticulatus* exhibited scars corresponding to the scutal areolae (Fig. 3.3C). A single set of exuviae of *S. bulla* exhibited what could be scars associated with scutal areolae, but the orientation of the specimen made interpretation difficult.

Functional Morphology

No pores were observed on the scutal areolae externally in *A. mcmillani, C. hollensis, L. americanus,* and *S. thomsenae* (Fig. 3.1, 3.2, 3.3A) or internally in *C. hollensis* (Fig. 3.4B), excluding the possibility that these structures have a secretory function. Internal examination of the structures did not reveal any apodemes (Fig. 3.4). No muscle tissue or nerve tissue was associated with the scutal areolae in *C. haematopotus* (Fig. 3.5) or *C. hollensis*.

When light was shined on the scutal areolae of *C. spinosus* at various intensities reflectance was observed (Fig. 3.6). Even at low intensities of light, scutal areolae showed reflective properties (Fig. 3.6D). Lesser angles of light produced more reflectance than greater angles in *A. mcmillani* (Fig. 3.7) and *C. spinosus*. Reflectance was observed at low levels when the light source was positioned dorsally (Fig. 3.7A) and at greater levels when positioned laterodorsally (Fig. 3.7D), laterally (Fig. 3.7E), and posteriorly (Fig. 3.7G). No reflectance was observed when the light was positioned anterodorsally (Fig. 3.7B), anteriorly (Fig. 3.7C), or posterodorsally (Fig. 3.7F).

Characters and Phylogeny

Character 82: Scutal areolae absent (plesiomorphic), scutal areolae present (apomorphic).

Character 83: Scutal areolae flush with cuticle of scutum or absent (plesiomorphic), scutal areolae distinctly raised above the cuticle of scutum, nodule-like (apomorphic).

The major clades of Borkent (2000) and Borkent and Craig (2004) were recovered in the phylogenetic reconstruction (Fig. 3.8). The Culicoidini were sister to all other Ceratopogoninae and a synapomorphy was found for *Culicoides* and *Paradasyhelea.* The genus *Ceratopogon* was not recovered as the sister to the remaining Ceratopogoninae (non-Culicoidini), but the *Ceratopogon*, *Stilobezzia, Fanthamia,* and *Macrurohelea* formed an unresolved polytomy sister to the remaining Ceratopogoninae (Fig. 3.8).

Discussion

Previous authors have proposed various names (e.g., pits, pores) and functions (e.g., sensory) for the scutal areolae of Ceratopogonidae (Tokunaga 1937, Wirth and Blanton 1959, Wirth and Hubert 1989), but the proposed names do not accurately describe the structures and proposed functions are incorrect. These structures just anterior to the scutellum are neither pores nor pits, but smooth cuticle that is flush with or raised above the surrounding cuticle. No pores are present on the surface excluding the function of secretion and no nerves are associated internally excluding sensory function. The scutal areolae are not muscle scars as no muscle tissue is associated with them. A probable hypothesis is that these scutal areolae function in communication among

individuals by reflecting light. The reflectant properties of the scutal areolae at low light angles and intensities indicate a possible role in swarming and mating. Males of *A. mcmillani* swarm early in the morning (6:45-9:15 am) (Borkent and Craig 2004) when the angle and intensity of light are lower. The reflected light from the scutal areolae might help males maintain spacing within swarms and recognize females entering swarms. The raised scutal areolae of *Culicoides* might have been selected to enhance reflectance in lower light conditions. Species of *Culicoides* swarm at crepuscular times (Downes 1955), and the scutal areolae elevated above the microtrichia would hypothetically receive and reflect more light than scutal areolae that are flush with the cuticle at low light angles.

The similarity in form and location indicates that the scutal areolae in various genera of Ceratopogonidae are homologous. The most parsimonious explanation of the origin of these structures is that they evolved once in the ancestor of ceratopogonids and were lost on two independent occasions in the Forcipomyiinae+Dasyheleinae and in the majority of the Ceratopogoninae. The reason(s) for such losses are unclear. If these structures function in swarming and mating, perhaps changes in these behaviors in the Forcipomyiinae+Dasyheleinae and Ceratopogoninae eliminated the need for these structures. A more sound understanding of the role of the scutal areolae is needed to address possible selection pressures for the origin and losses of these structures.

Characters 82 and 83 describing the presence-absence and raised or flushed condition of scutal areolae, respectively, provide improved resolution of relationships among ceratopogonid genera. No single, strong synapomorphy has been presented for the

genus *Culicoides* or the tribe Culicoidini. The raised state of the scutal areolae in *Culicoides* and *Paradasyhelea* provides the first synapomorphy for any genera in this tribe. Closer examination of *Washingtonhelea* might reveal raised scutal areolae in this genus and thus provide a synapomorphy for the entire tribe Culicoidini. Within the tribe Ceratopogonini, the generic relationships are largely unresolved. The unresolved polytomy of *Ceratopogon+Fanthamia+Macrurohelea+Stilobezzia* could be resolved by further examination of the scutal areolae. The scutal areolae in *Stilobezzia* are staggered, with one nodule more anterior than the other. The scutal areolae of *Ceratopogon, Fanthamia,* and *Macrurohelea* could have a staggered placement as the similarities have been noted among the four genera of this polytomy (Wirth 1965, Grogan and Wirth 1979, Grogan and Wirth 1985, de Meillon 1939). In addition to morphological similarities, a close relationship among *Ceratopogon, Fanthamia,* and *Macrurohelea* is biogeographically logical. *Ceratopogon* is Holarctic in distribution, *Fanthamia* is Ethiopean, and *Macrurohelea* is New Tropical and Australian. Detailed study of these genera might reveal synapomorphies confirming their relatedness.

The scutal areolae of Ceratopogonidae are likely more phylogenetically informative than the two synapomorphies presented here. The number, spacing, and placement of the scutal areolae are potentially informative phylogenetically. Four scutal areolae are present in *L. americanus* (Fig. 3.2 A, B) and *L. torrens* (Townsend), 1893, and the scutal areolae of *A. mcmillani* are bilobed (Figure 3.2D). This character could not be polarized, as scutal areolae are not present in the outgroup Chironomidae (P. Cranston, personal communication). However, the fossil genus *Lebanoculicoides*, which is sister to

all other Ceratopogonidae (Borkent and Craig 2004), can be examined and possibly allow the character states to be polarized. The spacing of the scutal areolae could be polarized in the same manner. In *L. americanus* and *A. mcmillani,* the scutal areolae are widely spaced; in *Culicoides,* the scutal areolae are closely spaced; and in *Ceratopogon* and *Stilobezzia* the scutal areolae are nearly touching. If these scutal areolae are present and visible in *Lebanoculicoides*, the character states could be polarized.

The occurrence of these scutal areolae in fossil specimens could provide further resolution to the phylogeny of Ceratopogonidae. Scutal areolae are predicted to be in *Minyohelea, Jordanoconops, Archiaustroconops, Fossileptoconops,* and *Protoculicoides* based on their phylogenetic proximity to *Leptoconops* and *Austroconops* (Borkent and Craig 2004). The presence of scutal areolae also is predicted for *Adelohelea* and *Heleageron*, which group near members of the Culicoidini (Borkent 2000). The presence of scutal areolae in the extinct genera of the Ceratopogonini is difficult to predict. *Brachycretacea, Paleobrachypogon,* and *Peronehelea* are found in amber deposits during the same timeframe as fossil *Stilobezzia* and might indicate the likelihood of scutal areolae being present in these fossil specimens.

The scutal areolae of Ceratopogonidae are unique structures among the Diptera. The current evidence suggests role in communication among individuals by reflecting light and are potential components of swarming and mating. Further evaluation of the morphology of these structures will further elucidate their function. Further evaluation of these structures in a phylogenetic context will provide greater resolution among the relationships of extant and extinct genera of Ceratopogonidae.

References

Borkent, A. 1995. Biting midges in the Cretaceous amber of North America (Diptera: Ceratopogonidae). Leiden: Backhuys, 237 pp.

Borkent, A. 2000. Biting midges (Ceratopogonidae: Diptera) from Lower Cretaceous Lebanese amber with a discussion of the diversity and patterns found in other ambers. *In* D. Grimaldi (editor), Studies on fossils in amber, with particular reference to the Cretaceous of New Jersey, pp. 355-451. Leiden: Backhuys.

Borkent, A. 2012. World Species of Biting Midges (Diptera: Ceratopogonidae). Illinois Natural History Museum (http://www.inhs.illinois.edu/research/FLYTREE/CeratopogonidaeCatalog.pdf). [accessed 28 February 2012].

Borkent, A. and D.A. Craig. 2004. *Austroconops* Wirth and Lee, a Lower Cretaceous genus of biting midges yet living in Western Australia: a new species, first description of the immatures and discussion of their biology and phylogeny (Diptera: Ceratopogonidae). *American Museum Novitates* No. 3449, 67 pp.

Borkent, A. and S. McKeever. 1990. First instar larvae of *Corethrella appendiculata* Grabham have a prothoracic proleg (Diptera: Corethrellidae). *Entomologica Scandinavica* 21: 219-223.

De Meillon, B. 1939. A new subgenus of *Ceratopogon*. Ruwenzori Expedition 1934-5 (British Museum of Natural History) 1: 103-107.

Downes, J.A. 1955. Observations on the swarming flight and mating of *Culicoides* (Diptera: Ceratopogonidae). *Transactions of the Royal Entomological Society of London* 106: 213-236.

Grogan, W.L., Jr., and W.W. Wirth. 1979. *Notiohelea*, a new genus of biting midges of the tribe Ceratopogonini from Chile (Diptera: Ceratopogonidae). *Pan-Pacific Entomologist* 54: 283-286.

Grogan, W.L., Jr., and W.W. Wirth. 1985. Two new Australian species of *Macrurohelea,* with a description of the male of *M. commoni* (Diptera: Ceratopogonidae). *International Journal of Entomology* 27: 128-135.

Page, R.D.M. 1996. Treeview: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358.

Sinclair, B.J. 1992. A new species of *Trichothaumalea* (Diptera: Thaumaleidae) from eastern North America and a discussion of male genitalic homologies. *Canadian Entomologist* 124: 491-499.

Tokunaga, M. 1937. Sand flies (Ceratopogonidae, Diptera) from Japan. *Tenthredo* 1:233-338.

Wirth, W.W. 1965. Two new species of *Macrurohelea* from Chile (Diptera, Ceratopogonidae). *Pan-Pacific Entomologist* 41: 46-50.

Wirth, W.W. and F.S. Blanton. 1959. Biting midges of the genus *Culicoides* from Panama (Diptera: Heleidae). *Proceedings of the United States National Museum* 109:237-482.

Wirth, W.W. and A.A. Hubert. 1989. The *Culicoides* of Southeast Asia (Diptera: Ceratopogonidae). *Memoirs of the American Entomological Institute* 44: 1-508.

Wood, D.M. and A. Borkent. 1989. Phylogeny and classification of the Nematocera. Ch. 114, pp. 1333-1370, *In* Manual Nearctic Diptera. Vol. 3. Agriculture Canada Monograph 32. pp. 1333-1581.

Zaman, V. 1983. Scanning Electron Microscopy of Medically Important Arthropods. Mauzen Asia, Singapore. 175 pp.

Table 3.1. Survey of species and sexes of Ceratopogonidae for the presence of scutal areolae.

Taxon	**Sex(es) Examined**	**Scutal Areolae Present**
Leptoconopinae		
Austroconops mcmillani	♀	Yes
Leptoconops americanus	♀, ♂	Yes
L. linsleyi	♀	Yes
L. torrens	♀	Yes
Forcipomyiinae		
Atrichopogon archboldi	♀, ♂	No
A. fusculus	♀, ♂	No
A. geminus	♀	No
A. levis	♀	No
A. maculosus	♀, ♂	No
A. websteri	♀, ♂	No
Forcipomyia cilipes	♀, ♂	No
F. fimbriata	♀, ♂	No
F. fuliginosa	♀, ♂	No
F. glauca	♀, ♂	No
F. pilosa	♂	No
F. quatei	♂	No
Dasyheleinae		
Dasyhelea cincta	♀, ♂	No
D. flavifrons	♀, ♂	No
D. major	♂	No
Ceratopogoninae		
Culicoidini		
C. baueri	♂	Yes
C. bermudensis	♀	Yes
C. debilipalpis	♀	Yes
C. furens	♀, ♂	Yes
C. haematopotus	♀	Yes
C. hollensis	♀, ♂	Yes
C. mississippiensis	♀	Yes
C. pallidicornis/niger	♀, ♂	Yes
C. sonorensis	♀, ♂	Yes
C. spinosus	♀	Yes
C. stellifer	♀	Yes
C. variipennis	♀, ♂	Yes
C. venustus	♀, ♂	Yes

Table 3.1. *Continued.*

C. villosipennis	♂	Yes
Paradasyhelea albpunctata	♀, ♂	Yes
P. minuta	♀, ♂	Yes
P. olympiae	♀, ♂	Yes
Washingtonhelea frommeri	♀	Yes
Ceratopogonini		
Allohelea johannseni	♂	No
A. nebulosa	♂	No
Alluaudomyia paraspina	♂	No
A. parva	♀	No
Atyphohelea macroneura	♀, ♂	No
Baeodasymia modesta	♀, ♂	No
Baeohelea nana	♀, ♂	No
Brachypogon canadensis	♀, ♂	No
B. laneae	♀, ♂	No
B. woodruffi	♀, ♂	No
Ceratoculicoides blantoni	♀	No
Ceratopogon abstrusus	♀, ♂	Yes
C. arcanus	♀, ♂	Yes
C. boomerangus	♀	Yes
C. culicoidithorax	♀	Yes
C. seculus	♀	Yes
C. willisi	♀	Yes
Downeshelea stonei	♀, ♂	No
Echinohelea lanei	♀, ♂	No
Fanthamia sp.	♀	Yes
Fittkauhelea amazonica	♀	No
Heteroceratopogon poguei	♀, ♂	No
Macrurohelea sp.	♀	Yes
Monohelea maculipennis	♀, ♂	No
Neurohelea granulosa	♀, ♂	No
Parabezzia spp.	♀, ♂	No
Serromyia crassifemorata	♀	No
Stilobezzia amnigena	♀, ♂	No
S. antennalis	♀, ♂	No
S. beckae	♀	Yes*
S. diminuta	♂	Yes
S. elegantula	♀, ♂	No
S. fuscula	♀, ♂	No
S. glauca	♀, ♂	No

Table 3.1. *Continued.*

S. guianae	♀, ♂	No
S. hirta	♀, ♂	Yes
S. lutea	♀, ♂	Yes*
S. kiefferi	♀, ♂	Yes
S. stonei	♀, ♂	Yes*
S. thomsenae	♀, ♂	Yes
Heteromyiini		
Clinohelea bimaculata	♀, ♂	No
C. curriei	♀, ♂	No
C. pseudonubifera	♀, ♂	No
Heteromyia fasciata	♀	No
H. pratti	♀	No
Jenkinshelea blantoni	♂	No
J. stonei	♂	No
Pellucidomyia wirthi	♀, ♂	No
Tetrabezzia pictipennis	♀	No
Sphaeromiini		
Austrosphaeromias apricans	♀, ♂	No
Mallachohelea atripes	♂	No
Nilobezzia schwarzii	♂	No
Probezzia xanthogaster	♂	No
Sphaeromias longipennis	♀, ♂	No
Palpomyiini		
Bezzia nobilis	♂	No
Palpomyia altispina	♂	No
P. basalis	♂	No
P. rubiginosa	♂	No
P. subaspera	♂	No
Phaenobezzia opaca	♂	No
Stenoxenini		
Paryphoconus lanei	♀	No
Stenoxenus johnsoni	♀	No

*Indicates that scutal areolae were present in some specimens and absent in others.

Table 3.2. Character numbers and associated sources in which characters are described that were used in the phylogenetic analysis.

Character	Source – character within source
1	Borkent and Craig (2004) – 1
2	Borkent and Craig (2004) – 2
3	Borkent and Craig (2004) – 3
4	Borkent and Craig (2004) – 4
5	Borkent and Craig (2004) – 5
6	Borkent and Craig (2004) – 6
7	Borkent and Craig (2004) – 7
8	Borkent and Craig (2004) – 8
9	Borkent and Craig (2004) – 9
10	Borkent and Craig (2004) – 10
11	Borkent and Craig (2004) – 11
12	Borkent and Craig (2004) – 12
13	Borkent and Craig (2004) – 13
14	Borkent and Craig (2004) – 14
15	Borkent and Craig (2004) – 15
16	Borkent and Craig (2004) – 16
17	Borkent and Craig (2004) – 17
18	Borkent and Craig (2004) – 18
19	Borkent and Craig (2004) – 19
20	Borkent and Craig (2004) – 20
21	Borkent and Craig (2004) – 21
22	Borkent and Craig (2004) – 22
23	Borkent and Craig (2004) – 23
24	Borkent and Craig (2004) – 24
25	Borkent and Craig (2004) – 25
26	Borkent and Craig (2004) – 26
27	Borkent and Craig (2004) – 27
28	Borkent and Craig (2004) – 28
29	Borkent and Craig (2004) – 29
30	Borkent and Craig (2004) – 30
31	Borkent and Craig (2004) – 31
32	Borkent and Craig (2004) – 32
33	Borkent and Craig (2004) – 34
34	Borkent and Craig (2004) – 35
35	Borkent and Craig (2004) – 36
36	Borkent and Craig (2004) – 37
37	Borkent and Craig (2004) – 38

Table 3.2. *Continued.*

38	Borkent and Craig (2004) – 39
39	Borkent and Craig (2004) – 40
40	Borkent and Craig (2004) – 41
41	Borkent and Craig (2004) – 42
42	Borkent and Craig (2004) – 43
43	Borkent and Craig (2004) – 44
44	Borkent and Craig (2004) – 45
45	Borkent and Craig (2004) – 46
46	Borkent and Craig (2004) – 47
47	Borkent and Craig (2004) – 48
48	Borkent and Craig (2004) – 49
49	Borkent and Craig (2004) – 50
50	Borkent and Craig (2004) – 51
51	Borkent and Craig (2004) – 52
52	Borkent and Craig (2004) – 53
53	Borkent and Craig (2004) – 54
54	Borkent and Craig (2004) – 55
55	Borkent and Craig (2004) – 56
56	Borkent and Craig (2004) – 57
57	Borkent (1995) – 28
58	Borkent (1995) – 29
59	Borkent (1995) – 30
60	Borkent (1995) – 31
61	Borkent (1995) – 32
62	Borkent (1995) – 33
63	Borkent (1995) – 34
64	Borkent (1995) – 35
65	Borkent (1995) – 36
66	Borkent (1995) – 37
67	Borkent (1995) – 38
68	Borkent (1995) – 39
69	Borkent (1995) – 40
70	Borkent (1995) – 41
71	Borkent (1995) – 42
72	Borkent (1995) – 43
73	Borkent (1995) – 44
74	Borkent (1995) – 45
75	Borkent (1995) – 46
76	Borkent (1995) – 48
77	Borkent (1995) – 49

Table 3.2. *Continued.,*

78	Borkent (1995) – 50
79	Borkent (1995) – 51
80	Borkent (1995) – 52
81	Borkent (1995) – 54
82	New character
83	New character

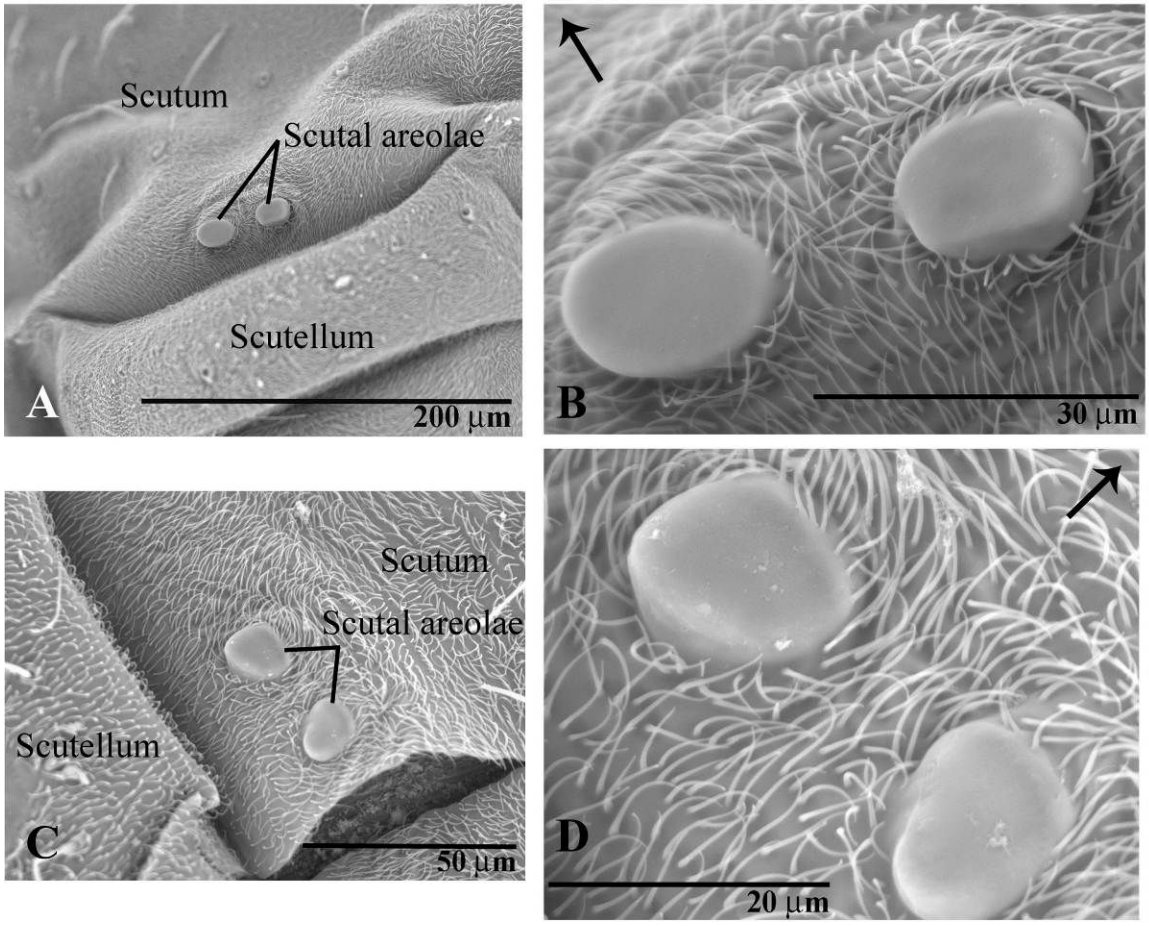


Figure 3.1. Scanning electron micrographs of *Culicoides hollensis* male and female. A: male scutum, scutellum, and scutal areolae, B: closeup of scutal areolae of male. C: female scutum, scutellum, scutal areolae, D: closeup of scutal areolae of female. Arrows indicate anterior end of midge.

Figure 3.2 (Next page). Scanning electronmicrographs of scutal areolae of Ceratopogonidae. A, B: male *Leptoconops americanus,* A: scutum, scutellum, and scutal areolae, B: closeup of scutal areolae. C, D: female *Austroconops mcmillani*, C: scutum, scutellum, and scutal areolae, D: closeup of scutal areolae. E, F: male *Stilobezzia thomsenae*, E: scutum, scutellum, and scutal areolae, F: closeup of scutal areolae. G, H: female *Bezzia nobilis*, G: scutum, and scutellum, H: closeup of scutum and scutellum. Arrows indicate anterior end of midge.

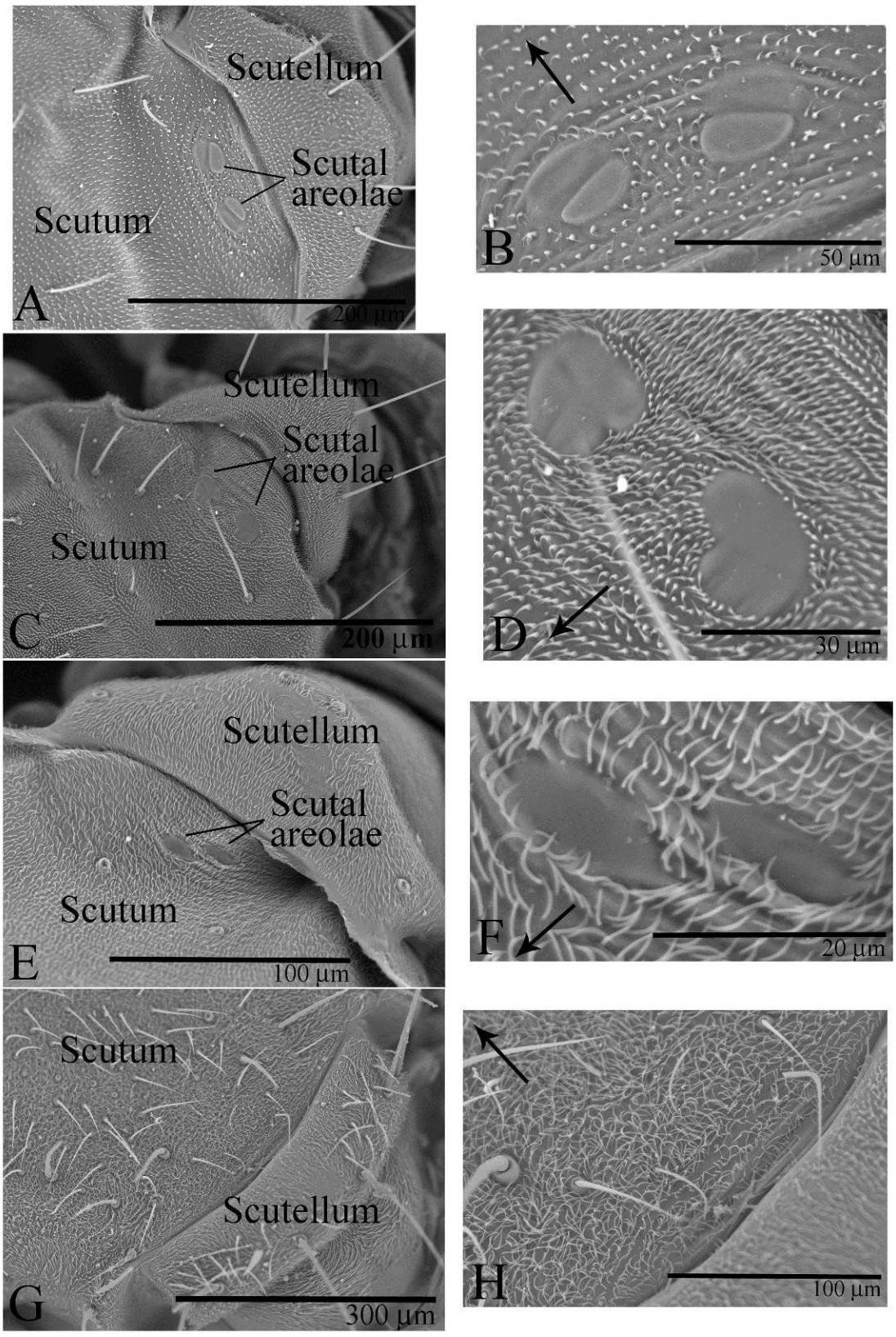
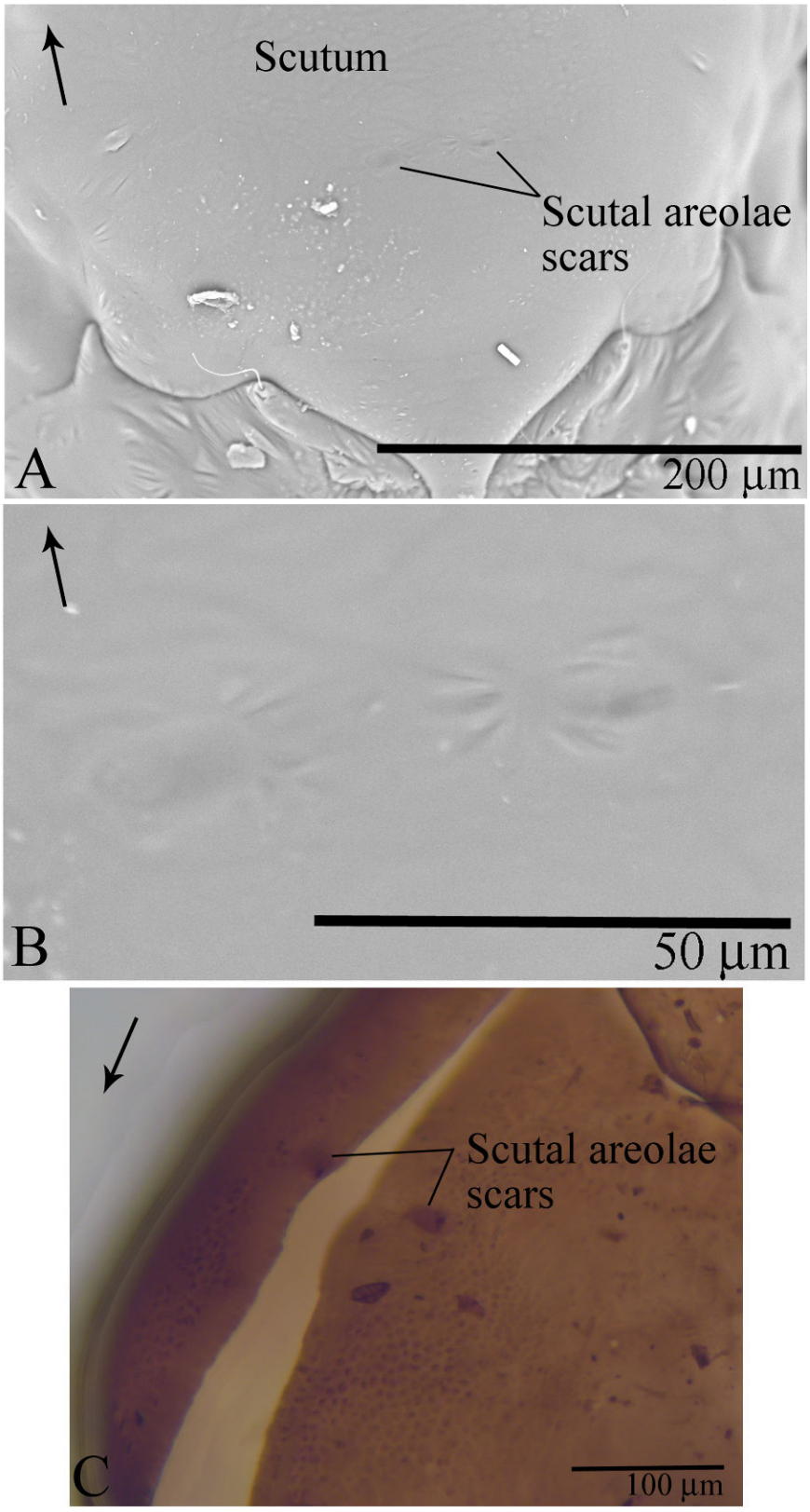

Scutellum
Scutal areolae
Scutum
A
200 µm
B
50 µm
Scutellum
Scutal areolae
Scutum
C
200 µm
D
30 µm
Scutellum
Scutal areolae
Scutum
E
100 µm
F
20 µm
Scutum
Scutellum
G
300 µm
H
100 µm

Figure 3.3 (Next page). Scars of scutal areolae of *Culicoides* pupae. A: Scanning electron micrograph of a pharate pupa of *C. guttipennis* showing scars of scutal areolae with scars marked. B: Closeup scanning electron micrograph of the scars of the scutal areolae, C. Light micrograph of the exuviaee of *C. denticulatus* with the scars of the scutal areolae marked. Arrows denote anterior ends of specimens.


Scutum
Scutal areolae scars
A
200 µm
B
50 µm
Scutal areolae scars
C
100 µm

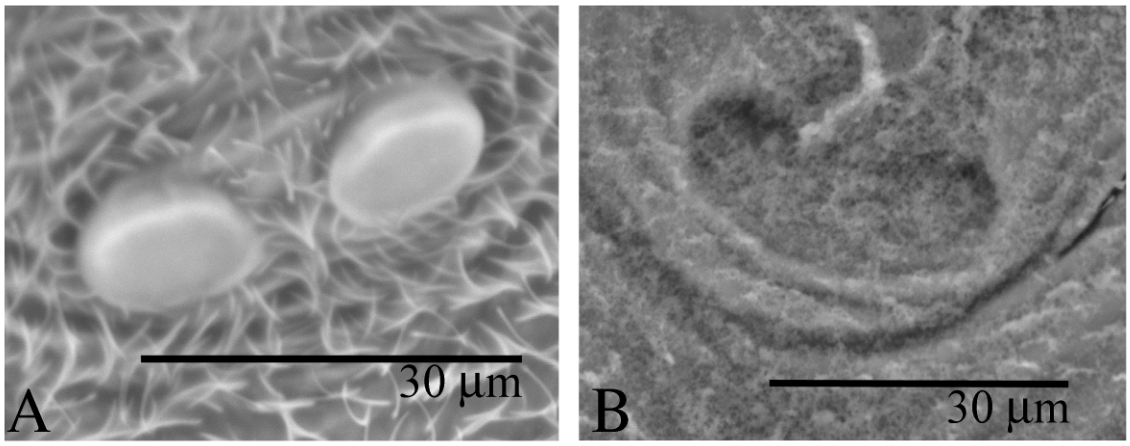


Figure 3.4. Variable pressure SEM images of the scutal areolae of a female *C. hollensis* without sputter coating. A: External view, B: Internal view (specimen was cleared in lactic acid prior to dehydration and imaging).

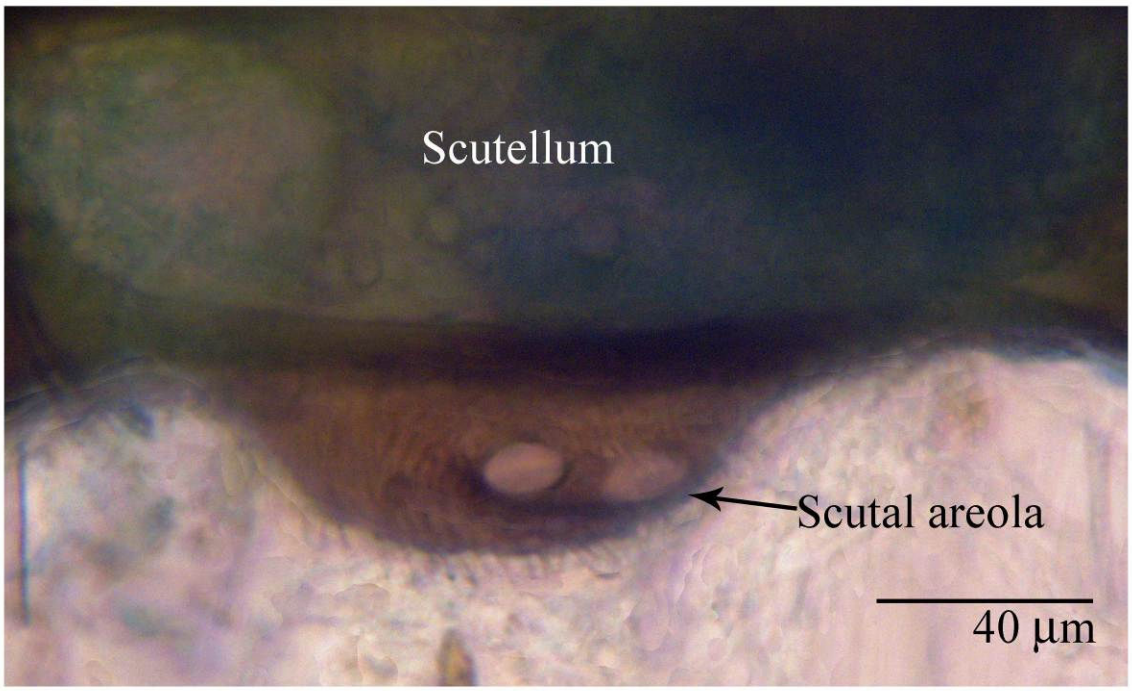


Figure 3.5. Light micrograph of an internal view of scutal areolae and scutellum dissected from *Culicoides haematopotus*. The scutum was removed during dissection, leaving only the prescutellar shield surrounding the scutal areolae. Tissue was stained in 0.025% methylene blue.

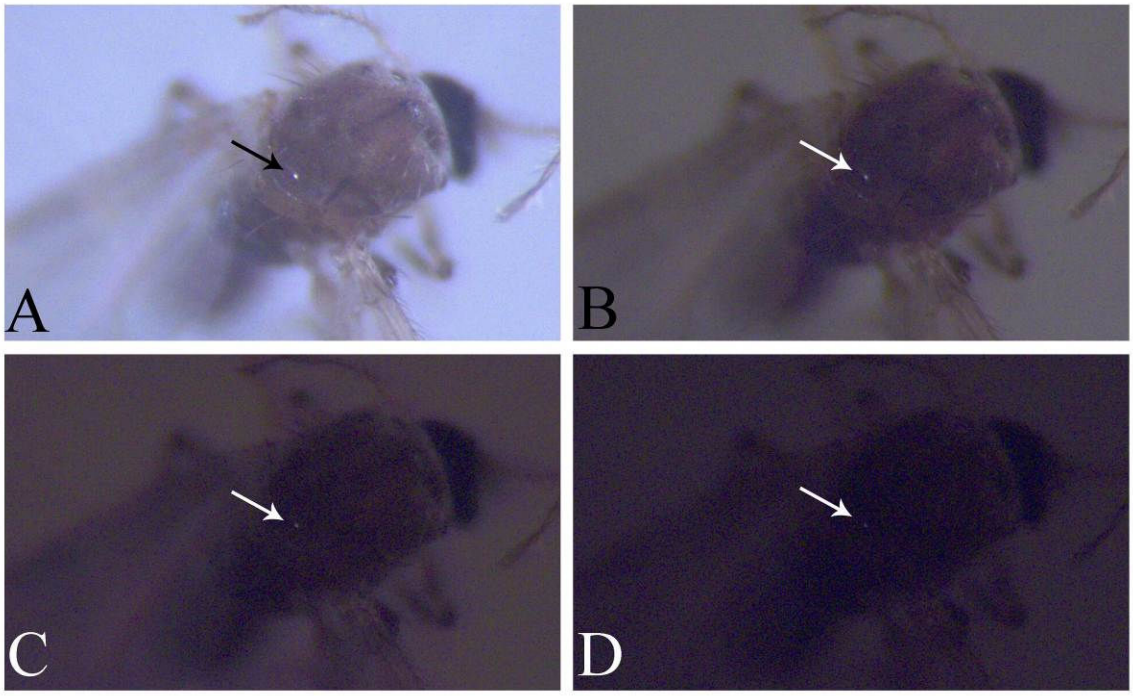


Figure 3.6. Reflectance of scutal areolae of female of *Culicoides spinosus* at various light intensities. Arrows indicate scutal nodule(s) reflecting light. Light intensities decrease from panel A-D.

Figure 3.7. (Next Page) Reflectant properties of scutal areolae on female of *Austroconops mcmillani* at various light angles. A: light source dorsal, B: light source anterodorsal, C: light source anterior, D: light source laterodorsal, E: light source lateral, F: light source posterodorsal, G: light source posterior. Arrows indicate scutal module(s) reflecting light.

A
B
C
D
E
F
G

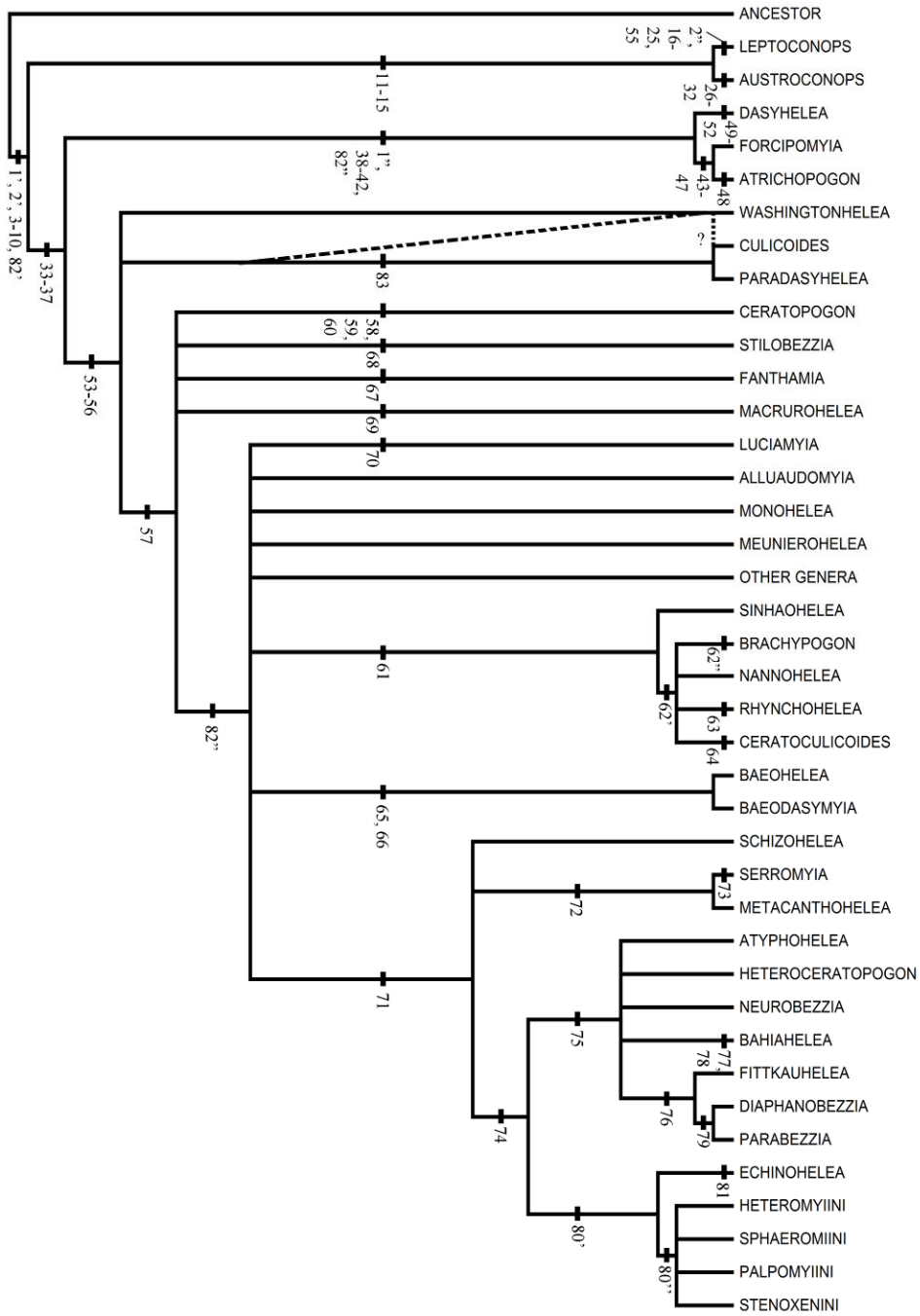


Figure 3.8. Phylogeny of Ceratopogonidae (genera and tribes) based on 83 morphological characters. The Ancestor group was the family Chironomidae and the group "Other genera" includes 27 genera (Borkent 2000).

CHAPTER FOUR

PHYLOGENETIC ASSESSMENT OF THE SUBGENERA OF NEARCTIC *CULICOIDES*

Introduction

Of the 6,089 species of described extant Ceratopogonidae, over 1,400 are of the genus *Culicoides* (Borkent 2012a). Species within the genus are of medical, veterinary, and economic importance (Borkent 2004, Mullen 2009). Despite the species richness and the medical, veterinary, and economic importance of the genus, little is known about the phylogenetic relationships of *Culicoides* species. No cladistics analysis has been conducted on the group, and no synapomorphies have been documented in support of the monophyly of the genus. Borkent (1995) proposed sensilla coeloconica on flagellomeres distad of the first flagellomere as a possible synapomorphy, but explained that the character appears in other genera, including the hypothesized sister genus *Paradasyhelea*.

The current subgeneric classification of *Culicoides* is based on phenetic similarities (Borkent 2012b). The number of subgenera, species groups, and species included within each subgeneric classification depends on the author. A conservative estimate of 31 subgenera and 38 unplaced species groups was provided by Borkent (2012b). Potential synapomorphies are discussed in the literature for the subgenera (e.g., *Avaritia* (Jamnback and Wirth 1963), *Hoffmania* (Blanton and Wirth 1979), *Selfia* (Atchley 1970)), but these characters have not been analyzed cladistically. Khalaf (1954) was one of the first to attempt to resolve the evolutionary relationships among members of *Culicoides*, but this work was phenetic. Since Khalaf (1954), ceratopogonid workers

continued to place species into subgenera and species groups based on overall similarity and little work was conducted on the relationships of these subgeneric groups. Outbreaks of bluetongue virus in southern Europe in 1998 and central Europe in 2006 and identification of novel vectors of the virus (Melhorn et al. 2007) have increased interest in the phylogenetic relationships of the genus. However, these studies have focused primarily on the species of the subgenera *Avaritia* and *Culicoides* (Linton et al. 2002, Dallas et al. 2003, Gomulski et al. 2005, Schwenkenbecher et al. 2009), which include known and suspected vectors of bluetongue virus in Europe (Melhorn et al. 2007).

The genus *Culicoides* is in need of systematic revision. There is no consensus on the subgeneric classification of the genus. Ceratopogonid workers construct subgenera and species groups based on local faunas rather than on the global fauna, resulting in taxonomic chaos for the group (Borkent 2012b). The goal of my study was to assess the phylogenetic relationships of the subgenera of Nearctic *Culicoides* for a framework of a future global study of the genus.

Materials and Methods

Species of Nearctic *Culicoides* were selected for phylogenetic analysis from each of the 13 subgenera and 7 species groups (Borkent and Grogan 2009) (Table 4.1). One to three species were selected from each subgenus and species group. Species were selected based on availability of descriptions of the male. An effort was made to include species from different geographic regions and previously proposed groupings. For the outgroup comparison, 3 species of *Paradasyhelea*, *Washingtonhelea frommeri* Wirth and Grogan, 1988, and an ancestor taxon of each of the genera *Forcipomyia, Atrichopogon*, and

Dasyhelea were included (Table 4.1). Morphological characters were obtained from illustrations and descriptions in the literature (Table 4.2). When available, slide-mounted specimens were examined to confirm illustrated and described characters (Table 4.2). All characters were coded as unordered with equal weights. Relationships among taxa were inferred by performing a heuristic search using parsimony analysis and a tree-bisection reconnection algorithm in PAUP* 4.0b10 (Sinauer Associates, Inc., Sunderland, MA). A majority rule consensus tree (50%) was calculated and printed using Treeview (Page 1996) and labeled using Adobe Photoshop Elements (Adobe Systems Inc., San Jose, CA).

Results

Thirty-one morphological characters were coded for 38 species of Nearctic *Culicoides* and 5 outgroup taxa (Table 4.2). All characters were parsimony informative.

Morphological Characters

1. Swimming motion of larvae slow, thrashing, not serpentine (plesiomorphic); swimming motion rapid, serpentine motion (apomorphic).

This character was discussed by Borkent and Craig (2004: char. 54) as a synapomorphy for the Ceratopogoninae (Fig. 4.1).

2. Posterior proleg of larva present (plesiomorphic); posterior proleg absent.

This character was discussed by Borkent and Craig (2004: char. 55) as a synapomorphy for the Ceratopogoninae (Fig. 4.1).

3. Anterior portion of abdominal tergite 4 of pupa with no more than one seta and one pore (plesiomorphic); anterior portion of abdominal tergite 4 with two setae (apomorphic).

This character was discussed by Borkent and Craig (2004: char. 56) as a synapomorphy for the Ceratopogoninae (Fig. 4.1).

4. Sternite 9 of female abdomen complete ventrally (plesiomorphic); sternite 9 incomplete ventrally, dividing the sternite in two (apomorphic).

This character was discussed by Borkent (1995: char. 26) as a synapomorphy for the Ceratopogoninae (Fig. 4.1), although the status of the character in *Washingtonhelea* was questionable.

5. Scutal areolae flush with surrounding cuticle of scutum or absent (plesiomorphic), Scutal areolae raised above surrounding cuticle, nodule-like (apomorphic).

This character was discussed in Chapter 3 as a synapomorphy of *Culicoides+Paradasyhelea* (Fig. 4.1). The state of this character could not be assessed in *Washingtonhelea* because of lack of specimens for imaging with scanning electron microscopy. This character could be a synapomorphy for all of the Culicoidini.

6. Sensilla coeloconica present on only flagellomere 1 (plesiomorphic), sensilla coeloconica present on flagellomere 1 and additional flagellomeres (apomorphic).

Borkent (1995) included this character as a synapomorphy of *Culicoides* (char. 27), and he also included the possibility of this character being a synapomorphy for *Culicoides+Paradasyhelea,* as some species of *Paradasyhelea* have sensilla coeloconica on more than first flagellomere. This character also occurs in the genera *Austrohelea, Brachypogon*, *Fanthamia,* and *Macrurohelea* and patterns of flagellomeres bearing

sensilla coeloconica frequently change within the *Culicoides,* indicating the complexity of interpreting this character (see Borkent 1995 for further discussion).

7. Supraorbital suture in adult female absent (plesiomorphic), Supraorbital suture present above interocular seta as straight or arched line (apomorphic'), supraorbital suture present, inverted Y-shape (apomorphic").

This character is not observed in the subfamilies sister to the Ceratopogoninae (e.g., Leptoconopinae, Forcipomyinae, and Dasyheleinae). However, within the Ceratopogonidae, this character might have been acquired or lost on multiple occasions. Three of eleven genera of Ceratopogoninae examined have a supraorbital suture: *Culicoides, Echinohelea,* and *Stilobezzia*. Given the phylogenetic relationships of these three genera (Chapter 3, Borkent 2000), this character was likely gained independently within these lineages. However, the Y-shaped supraorbital suture of some *Culicoides* is unique. This character formed a synapomorphy for *C. palmerae* James, 1943, group+*C. travisi* Vargas, 1949 (Fig. 4.1). *Culicoides niger,* Root and Hoffman, 1939, and other species of the *C. stonei* group also have a Y-shaped supraorbital suture. Given the close proximity of *C. niger* to the *C. palmerae* group+*C. travisi* clade (Fig. 4.2), *C. niger* and other species of the *C. stonei* group might be a part of this clade.

8. Ventral root of gonocoxite absent, dorsal root may be present (plesiomorphic), ventral root present, simple, similar in shape to dorsal root (apomorphic'), ventral root present, anterior end foot-shaped (apomorphic").

Within the Ceratopogonidae, a dorsal root extending from the base of the gonocoxite is typically present, occasionally being reduced or absent in some taxa. The

ventral root of the gonocoxite is unique to *Culicoides* and is a synapomorphy for the genus exclusive of the subgenus *Selfia* (Fig. 4.1). Blanton and Wirth (1979) describe the ventral root as absent in *C. insignis* Lutz, 1913, and *C. venustus* Hoffman, 1925, but their illustration of *C. venustus* shows two small protuberances on the gonocoxite. One of these is the dorsal root; the other could be the ventral root or an artifact. Examination of the actual specimens is needed to assess if these characters are present or if a reversal to the plesiomorphic state occurred in these species. The anterior end of the ventral root is further modified in the subgenera *Diphaomyia, Haematomyidium,* and *Oecacta* and the *C. piliferus, C. leoni,* and *C. mohave* species groups into a foot-shaped structure and is a synapomorphy for these taxa. This modification could be coded as an ordered character transition but was left as an unordered character to reduce assumptions.

9. Parameres articulating with apodemes extending from tergum IX and dorsal root (plesiomorphic); apodemes extending from tergum IX absent, parameres articulating with the dorsal root or dorsal and ventral roots (apomorphic).

The parameres of *Leptoconops* and *Forcipomyia* articulate with apodemes extending from abdominal tergite IX. A similar condition is observed in *Dasyhelea,* thought interpretation of the character in this genus is complicated because of modification of the parameres. The parameres of *Washingtonhelea frommeri* are articulated via apodemes, as are the fused parameres of *Paradasyhelea*. In *Culicoides* exclusive of the subgenus *Selfia*, these apodemes are absent and the parameres articulate with the dorsal root or the dorsal and ventral roots. These apodemes are absent in multiple genera of the Ceratopogoninae, bringing to question the strength of this

character as a synapomorphy for *Culicoides*. However, the hypothesized sister relationship of the Culicoidini with other Ceratopogoninae (Chapter 3, Borkent 2000) and the presence of the apodemes in *Washingtonhelea* and *Paradasyhelea* indicate the absence of the apodemes of tergite IX is a synapomorphy for the *Culicoides* exclusive of the subgenus *Selfia* (Fig. 4.1).

10. Parameres divided (plesiomorphic); parameres fused into an inverted U with ends of U directed posteriorly (apomorphic'); parameres fused into a single triangular plate (apomorphic'').

The parameres of Ceratopogonidae have undergone switches between separate and fused parameres. Separate parameres occur in *Leptoconops, Forcipomyia, Dasyhelea,* and *Washingtonhelea*, and fused parameres occur in *Austroconops* and *Paradasyhelea*. Within the *Culicoides*, fused and separate parameres occur in multiple lineages. The apomorphic condition of the parameres fused into a small, inverted U or triangular plate is a synapomorphy of *Paradasyhelea*, though this is not reflected in the majority rule consensus tree (Fig. 4.1) due to multiple characters that could not be coded (Table 4.3).

11. Single spermatheca present (plesiomorphic); two or three spermathecae present (apomorphic).

This character is highly homoplasious. The number of spermathecae changes frequently in multiple genera throughout the Ceratopogonidae.

12. Spermatheca(e) spherical, ovoid, or pear shaped (plesiomorphic); spermatheca U-shaped (apomorphic'); Spermathecae elongate tubes, with little sclerotization (apomorphic").

The typical shape of the spermathecae throughout the Ceratopogonidae is spherical, ovoid, or pear-shaped. The modified U-shaped spermathecae (apomorphic') of the subgenus *Monoculicoides* is a synapomorphy for the group (Fig. 4.1). The three elongate, poorly sclerotized spermathecae of the subgenus *Selfia* is a unique and defining character for the group (Fig. 4.1).

13. Operculum of pupa without process near posterior margin (plesiomorphic); operculum of pupa with elongate process near posterior end (apomorphic).

This character is found in the subgenus *Hoffmania* and is not observed in any other Nearctic taxa (Fig. 4.1).

14. Operculum of pupa with spinules short (plesiomorphic); operculum with spinules elongate and hair-like (apomorphic).

The operculum of the pupae of *Culicoides* is covered with short, stout spinules, the arrangement of which can be diagnostic for species. In the subgenus *Avaritia*, these spinules are modified into long, hair-like structures. These modified spinules are a synapomorphy for the subgenus (Fig. 4.1).

15. Pupa with anterodorsal seta single (plesiomorphic); pupa with anterodorsal setae double (apomorphic).

The apomorphic condition is unique to the subgenus *Avaritia* (Fig. 4.1).

16. Pupa without a series of erect spines along inner margin of caudal apicolateral processes, at most a series of appressed scales (plesiomorphic); inner margin of caudal apicolateral processes with a series of erect spines (apomorphic).

The apomorphic condition is unique to the subgenus *Avaritia* (Fig. 4.1).

17. Mesal area of parameres unaltered, straight (plesiomorphic); mesal area of parameres with a lobe or large swelling (apomorphic).

In some species of *Culicoides,* the parameres are modified at midlength with a lobe or large swelling. This condition was observed in the subgenera *Diphaomyia* (except in the *C. baueri* group), *Haematomyidium,* and *Oecacta* and the *C. leoni* and *C. mohave* species groups (Fig. 4.1).

18. Shoulders of aedeagus lacking posteriorly directed processes (plesiomorphic); Shoulders of aedeagus with strong processes directed posteriorly (apomorphic).

Several species of *Culicoides* have processes projecting from the aedeagal sclerite in addition to the medial process, but these are likely not homologous. This character refers to the two spur-like processes projecting posteriorly from the shoulders of the aedeagus and not the medial process. This character is restricted to members of the subgenus *Diphaomyia* and is a synapomorphy for the group (Fig. 4.1).

19. Base of parameres straight, simple (plesiomorphic); base of parameres sigmoidal or boot-shaped (apomorphic'); base of parameres L-shaped, not sigmoidal, angle broad, expanded, plate-like in some taxa (apomorphic"); parameres fused (apomorphic"'), base of parameres straight with basal knobs (apomorphic"").

This character is difficult to interpret because of multiple modifications to the parameres of Ceratopogonidae (see discussion of character 10). However, the apomorphic" and the apomorphic"" conditions are phylogenetically informative. L-shaped, non-sigmoidal parameres occur in the subgenera *Avaritia* and *Hoffmania* (Fig. 4.1). In some species (e.g., *C. obsoletus* (Meigen), 1818*, C. insignis, C. venustus*), the angle is broadly expanded and plate-like. Straight parameres were coded as the plesiomorphic condition, but the bases of the parameres were of equal thickness with the mesal shaft of the parameres in the outgroup taxa. In some species of *Culicoides*, the bases of the parameres are noticeably thicker than the rest of the parameres forming a knob-like base. This character state was observed in the subgenera *Diphaomyia, Haematomyidium,* and *Oecacta;* the *C. piliferus, C. leoni,* and *C. mohave* species groups; and *C. stilobezzioides* Foote and Pratt, 1954, and *C. luglani* Jones and Wirth, 1958 (Fig. 4.1).

20. Wing without pale markings crossing vein M_1 (plesiomorphic); wing with vein M_1 bisecting a pale spot (apomorphic).

Wing markings are one of the most-used characters to group species of *Culicoides* into subgeneric groups. As with much of the previous work on *Culicoides*, these groupings are entirely phenetic. Polarizing wing markings is difficult, as markings can vary widely within subgenera and species.

21. Wing without pale markings crossing vein M_2 (plesiomorphic); wing with vein M_2 bisecting a pale spot (apomorphic).

See discussion of character 20.

22. Post stigmatic pale spot (pale spot distal of costa) absent (plesiomorphic); post stigmatic pale spot present, distal tip of costa and part of 2nd radial cell included in spot (apomorphic'); post stigmatic pale spot present, distal tip of costa and radial sector not included in spot (apomorphic").

This is one of the more consistent wing-mark characters. *Culicoides* have three classes of wing markings. The first is no wing markings; this is a character shared with *Washingtonhelea* and *Paradasyhelea* and is the plesiomorphic condition. The second condition is two pale spots, one over the r-m crossvein and one on the distal end of the costa or just distad of the costa known as the post stigmatic spot. The third class of wing patterns has the same conditions as class two plus additional spots on the remainder of the wing. The first and third conditions vary among species, with wing pale spots being absent and present in various locations. The post stigmatic spot is more consistent and has the potential to be phylogenetically informative. The pale spot lying on the distal tip of the costa and part of the 2nd radial cell occurs in the subgenera *Avaritia, Culicoides,* and *Hoffmania* and is a synapomorphy for the group. The condition also occurs in *C. hollensis, C. mississippiensis* Hoffman, 1926*,* and *C. spinosus*, but varies within each species with both apomorphic conditions occurring.

23. Rudimentary spermatheca absent (plesiomorphic); rudimentary spermatheca present (apomorphic).

In some *Culicoides*, a non-functional spermatheca can become sclerotized. This rudimentary spermatheca is visible in slide mounted specimens. This character was observed in all *Culicoides* exclusive of the subgenera *Selfia, Monoculicoides,* and

Beltranmyia and is a synapomorphy for the group. A third spermatheca is present in the subgenus *Selfia*, but the spermatheca is larger, unsclerotized, and the character state was not considered homologous.

24. Common oviduct without sclerotized ring (plesiomorphic); common oviduct with a sclerotized ring (apomorphic).

This character has some homoplasy. It was found in all species of the clade sister to *C. melleus* (Coquillett), 1901, and *C. niger*, with the exceptions of *C. spinosus* and *C. stilobezzioides*.

25. Basal arms of parameres without folded lobes directed anteriorly (plesiomorphic); basal arms of parameres with a lobe folded anteriorly (apomorphic).

This character is unique to the species *C. cacticola* Wirth and Hubert, 1960, and *C. copiosus* Root and Hoffman, 1937. Folded lobe of the parameres was not found in *C. hinmani* Khalaf, 1952, another hypothesized member of the subgenus *Drymodesmyia*.

26. Apicolateral processes of male tergum IX elongate (plesiomorphic); apicolateral processes of male tergum IX short or absent (apomorphic).

Elongate apicolateral processes in male Ceratopogonidae is a synapomorphy for the family (Borkent and Craig 2004), but short processes do occur in multiple lineages of Ceratopogonidae. In the Nearctic *Culicoides*, short apicolateral processes occur in the subgenera *Avaritia, Culicoides,* and *Hoffmania* and are a synapomorphy for the group.

27. Caudal margin of male tergum IX convex (plesiomorphic); caudal margin of male tergum IX transverse or transverse with a distinct medial notch (apomorphic).

The plesiomorphic condition of a rounded tergum IX was observed in the *Forcipomyia, Atrichopogon,* and *Leptoconops*. In the nearest sister groups to the genera *Culicoides*, *Washingtonhelea* and *Paradasyhelea*, the caudal margin of tergum IX is transverse or transverse with a distinct medial notch. This is the typical character state in the Nearctic *Culicoides,* except for the subgenera *Avaritia* and *Culicoides*, which exhibit the plesiomorphic state.

28. Parameres composed of two rods or fused without a pair of posterior projections (plesiomorphic); parameres fused with a pair of processes directed posterad (apomorphic).

Among the Nearctic *Culicoides*, this character is restricted to the subgenus *Monoculicoides*.

29. Distal ends of parameres smooth, without spines (plesiomorphic); distal ends armed with fringe of spines or teeth (apomorphic).

The crown of spines at the distal tips of the parameres of *C. spinosus* are likely not homologous to the fringe of spines in other species. Furthermore, the armature at the distal end of the parameres in the subgenera *Diphaomyia* and *Oecacta* are modified into large teeth. The fringe of spines observed in some taxa could be an intermediate state between no spines on the parameres and teeth, or these character states could have arisen independently. Examination of actual specimens could help resolve the states of this character. This character is congruent with characters 8 and 19, supporting phylogenetic relationships among the subgenera *Diphaomyia, Haematomyidium,* and *Oecacta* and the *C. piliferus, C. leoni,* and *C. mohave* species groups (Fig. 4.1).

30. Sensilla coeloconica absent from distal flagellomeres (plesiomorphic); sensilla coeloconica present on at least some of flagellomeres 2-8 only (apomorphic'); sensilla coeloconica present on at least some of flagellomeres 9-13 only (apomorphic''); sensilla coeloconica present on at least some of flagellomeres 2-8 and flagellomeres 9-13 (apomorphic''').

The patterns of sensilla coeloconica have been frequently used by ceratopogonid workers to established subgenera and species groups of *Culicoides*. Interpretation of this character is complex, as already discussed (character 6). Even though there is a high likelihood of homoplasy, this character can be phylogenetically informative at less inclusive levels.

31. Distal end of parameres tapered to tip (plesiomorphic); distal end of parameres broadly expanded, bearing large teeth (apomorphic).

The expanded ends of the parameres with large teeth were restricted to species of *Diphaomyia* (Fig. 4.1). Similar character states were observed in species of *Oecacta*, which also had large teeth on the parameres but still extended to a tapered tip. This character state might be an intermediate state between the plesiomorphic and apomorphic conditions. Examination of actual specimens will help in the interpretation of this character.

Phylogeny

The heuristic search resulted in 6048 equally parsimonious trees with tree lengths of 65. The ensemble consistency index (CI) was 0.631, the ensemble rescaled consistency index (RCI) was 0.545, and the ensemble retention index (RI) was 0.864. Five characters,

11, 21, 27, 29, and 30, had a consistency index (ci) less than 0.400; two characters, 8 and 22, had a ci of 0.400; and three characters, 7, 17, and 20, had a ci of 0.500. All other characters had a ci of equal to or greater than 0.667. Excluding characters 11, 21, 27, 29, and 30 resulted in 9381 equally parsimonious trees with lengths of 44, CI of 0.773, RCI of 0.706, RI of 0.913, and little change to the topology of the majority rule (50%) consensus tree (Fig. 4.2). One difference in topology between consensus trees was *C. melleus*, *C. niger*, and *C. palmerae+C.oregonensis+C. travisi* became part of the large polytomy that contained eight clades, with *C. crepuscularis* Malloch, 1915, and *C. hollensis* becoming sister to this new polytomy. Another difference was the removal of *C. spinosus* and *C. stilobezzioides* from the clade including the subgenera *Diphaomyia, Haematomyidium,* and *Oecacta* and the *C. deadalus, C. piliferus, C. leoni,* and *C. mohave* species groups (Fig. 4.2).

Fourteen clades were found in 100% of the equally parsimonious trees (Fig. 4.2). The tribe Culicoidini (*Washingtonhelea+Paradasyhelea+Culicoides*) with *Paradasyhelea* sister to *Culicoides* was inferred in all trees. The genus *Culicoides* also was monophyletic. Within the *Culicoides,* the subgenus *Selfia* was sister to all other *Culicoides.* The subgenera *Avaritia, Diphaomyia, Hoffmania,* and *Monoculicoides* were all monophyletic (the subgenus *Selfia* also is monophyletic but only one species was included in the analysis). Other monophyletic groupings included the subgenera (*Avaritia+Hoffmania*)+*Culicoides*), the subgenus *Diphaomyia*+*C. piliferus* group, *Haematomyidium*+*Oecacta*+*C. leoni* group+*C. mohave* group, and *C. palmerae*

group+*C. travisi.* The subgenera *Drymodesmyia* and *Silvaticulicoides* and the *C. deadalus* species group were polyphyletic (Fig. 4.2).

To provide more resolution to the phylogeny, the clade defined by character 29 (Fig. 4.1) (i.e., *Diphaomyia+Haematomyidium+Oecacta+Withomyia+C. piliferus* group+*C. leoni* group+*C. mohave* group+*C. spinosus+C. luglani* clade) was assessed using maximum likelihood of COI sequences (Chapter 2). Only species for which COI sequences were available were used (Table 4.3). In addition to this clade, the clade defined by characters 22' and 26 (Fig. 4.1) (i.e., *Avaritia+Hoffmania+ Culicoides*) was included in the maximum likelihood analysis. This group was recovered in 100% of the maximum parsimony trees (Fig. 4.2) and served as an internal control. *Culicoides brookmani* Wirth, 1952, was used as the outgroup. Sequences were aligned using MUSCLE (Edgar 2004), and maximum likelihood analysis using a Tamura 3-parameter model with Gamma distributed and invariable sites for the first two codon positions with 100 bootstrap iterations was conducted in MEGA 5 (Tamura et al. 2011). The resulting tree grouped all specimens of the same species closely together except for *C. stellifer*, which was scattered throughout the tree (Fig. 4.3). The subgenus *Avaritia*, represented by *C. obsoletus* and *C. sanguisuga* (Coquillett), 1901, grouped closely with the subgenus *Hoffmania*, represented by *C.venustus,* as in the maximum parsimony analysis (Fig. 4.2 vs. Fig. 4.3).

The analysis was rerun with two clades analyzed in separate maximum likelihood analyses, using *C. brookmani* as the outgroup to try to resolve relationships among taxa defined by character 29. Because of the low number of sequences of Nearctic specimens

available for the *Avaritia+ Hoffmania+Culicoides* clade, sequences of Palearctic species available in GenBank were used to supplement the analysis (Table 4.4). Maximum likelihood analysis using a Hasegawa-Kishino-Yano model with Gamma distributed sites for the first two codon positions was conducted in MEGA 5 with 100 bootstrap iterations. The resulting bootstrap consensus tree (Fig. 4.4) recovered a similar topology to the maximum parsimony majority rule consensus tree (Fig. 4.2). Analysis of the clade defined by character 29 was conducted using maximum likelihood analysis with a Kimura 2-parameter model with Gamma distributed and invariable sites for the first two codon positions using MEGA 5 with 100 bootstrap iterations. Removal of the *Avaritia+ Hoffmania+Culicoides* clade did not improve resolution among the taxa (Fig. 4.5).

Discussion

The genus *Culicoides* is monophyletic with the potential synapomorphy for the genus being the presence of the supraorbital suture. Within the genus, the subgenus *Selfia* is sister to all other *Culicoides*. Species of this subgenus retain the plesiomorphic characters of the parameres articulating with apodemes extending from the ninth tergite and gonocoxite lacking a ventral root. In addition to these characters, members of the subgenus *Selfia* lack pale spots on the wings and have the sensilla coeloconica restricted to the first eight flagellomeres, as in the sister genus *Paradasyhelea*. The elongate, unsclerotized spermathecae of the species of *Selfia* is unique. The subgenus *Selfia* might not belong in the *Culicoides* and might need to be placed in a separate genus. However, such a conclusion is beyond the scope of this study, as a more thorough analysis including more species and life stages from all geographic areas is needed.

Maximum-likelihood analysis of the fragment of the COI gene for the clade composed of *Avaritia+Hoffmania+Culicoides* recovered a similar topology as the morphological analysis and as in other molecular studies (Linton et al. 2002, Dallas et al. 2003, Gomulski et al. 2005*,* Schwenkenbecher et al. 2009). However, the analysis did not improve resolution of the clade defined by character 29 (Fig. 4.2), confirming the unresolved polytomy of the morphological analysis. Sequences of the same species grouped together for all but two species. *Culicoides parapiliferus* Wirth and Blanton, 1974, had one specimen that did not group with other specimens of *C. parapiliferus*. *Culicoides stellifer* grouped in three different locations within the trees (Fig. 4.3, 4.5). These data indicate that *C. stellifer* is likely a species complex or has undergone introgression with other species of *Culicoides* causing misidentification by COI (Whitworth et al. 2007). The association of adult females of *C. stellifer* with larvae of distinctly different morphology (Chapter 2) gives weight to the species complex hypothesis. The addition of nuclear genes and additional morphological data are needed to help elucidate the unresolved polytomies and potential species complexes in *Culicoides*.

As predicted (Borkent 2012b), five subgenera and one species group of *Culicoides* were monophyletic, including *Avaritia, Diphaomyia, Hoffmania, Monoculicoides*, *Selfia,* and *C. palmerae* group+*C. travisi.* Also as predicted, some of the subgeneric groups were polyphyletic (3) or no supporting synapomorphies were found (10, only includes those taxa with more than 1 species analyzed). These results show the need for a systematic revision of the genus *Culicoides*.

The results of a phylogenetic analysis are only as good as the data entered into the matrix. Examining illustrations and descriptions of specimens has inherent limits, compared with examining actual specimens. Characters might have been overlooked or misinterpreted due to this methodology. Less than 3% of the global fauna of *Culicoides* was used in this study and only Nearctic fauna were included. More specimens from other geographic areas could provide greater resolution. However, the purpose of my study was to build initial hypotheses of the relationships among *Culicoides*. This methodology allowed for testable hypotheses to be built, which can be further investigated and expanded upon in future, more inclusive studies.

This study is the first cladistic analysis performed on the subgeneric classification of *Culicoides*. The analysis of 13 subgenera and 7 species groups of the Nearctic Region provides the framework for a phylogeny of *Culicoides* at a global scale. A global study of the phylogenetic relationships of *Culicoides* will be a valuable step toward stabilizing the taxonomy of the genus.

References

Atchley, W.R. 1967. The *Culicoides* of New Mexico (Diptera: Ceratopogonidae). *University of Kansas Science Bulletin* 46: 937-1022.

Atchley, W.R. 1970. A biosystematic study of the subgenus *Selfia* of *Culicoides* (Diptera: Ceratopogonidae). *University of Kansas Science Bulletin* 49: 181-336.

Blanton, F.S. and W.W. Wirth. 1979. The sand flies (Culicoides) of Florida (Diptera: Ceratopogonidae). Arthropods of Florida and Neighboring Land Areas 10. 204 pp.

Boesel, M.W. 1973. The genus *Atrichopogon* (Diptera, Ceratopogonidae) in Ohio and neighboring states. *Ohio Journal of Science* 73: 202-215.

Borkent, A. 1995. Biting midges in the Cretaceous amber of North America (Diptera: Ceratopogonidae). Leiden: Backhuys, 237 pp.

Borkent, A. 2000. Biting midges (Ceratopogonidae: Diptera) from Lower Cretaceous Lebanese amber with a discussion of the diversity and patterns found in other ambers. *In* D. Grimaldi (editor), Studies on fossils in amber, with particular reference to the Cretaceous of New Jersey, pp. 355-451. Leiden: Backhuys.

Borkent, A. 2004. Chapter 10. The biting midges, the Ceratopogonidae (Diptera). pp. 113-126. *In* Marquardt, W.C. (Ed.). Biology of Disease Vectors, 2nd Edition. Amsterdam, Elsevier Academic Press.

Borkent, A. 2012a. World Species of Biting Midges (Diptera: Ceratopogonidae). Illinois Natural History Museum (http://www.inhs.illinois.edu/research/FLYTREE/CeratopogonidaeCatalog.pdf). [accessed 28 February 2012].

Borkent, A. 2012b. The subgeneric classification of *Culicoides* – thoughts and a warning. Illinois Natural History Museum (http://www.inhs.illinois.edu/research/FLYTREE/CulicoidesSubgenera.pdf). [accessed 4 June 2012].

Borkent, A. and D.A. Craig. 2004. *Austroconops* Wirth and Lee, a Lower Cretaceous genus of biting midges yet living in Western Australia: a new species, first description of the immatures and discussion of their biology and phylogeny (Diptera: Ceratopogonidae). *American Museum Novitates* No. 3449, 67 pp.

Borkent, A. and W.L. Grogan, Jr. 2009. Catalog of the New World biting midges north of Mexico (Diptera: Ceratopogonidae). *Zootaxa* 2273: 1-48.

Chan, K.L. and E.J. LeRoux. 1971. Nine new species of *Forcipomyia* (Diptera: Ceratopogonidae) described in all stages. *Canadian Entomologist* 103: 729-762.

Dallas, J.F., R.H. Cruickshank, Y.-M. Linton, D.V. Nolan, M. Patakakis, Y. Braverman, R. Capela, I. Pena, R. Meiswinkel, M.D. Ortega, M. Baylis, P.S. Mellor, and A.J. Mordue (Luntz). 2003. Phylogenetic status and matrilineal structure of the biting midge, *Culicoides imicola*, in Portugal, Rhodes and Israel. *Medical and Veterinary Entomology* 17: 379-387.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792-1797.

Gomulski, L.M., R. Meiswinkel, J.-C. Delecolle, M. Goffredo, and G. Gasperi. 2005. Phylogenetic relationships of the subgenus *Avaritia* Fox, 1955 including *Culicoides obsoletus* (Diptera, Ceratopogonidae) in Italy based on internal transcribed spacer 2 ribosomal DNA sequences. *Systematic Entomology* 30: 619-631.

Grogan Jr., W.L. and R.A. Phillips. 2008. A new species of biting midge in the subgenus *Monoculicoides* of *Culicoides* from Utah (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 110: 196-203.

Grogan Jr., W.L. and E.A. Sigrist. 2007. A new Nearctic species of biting midge in the subgenus *Metaforcipomyia* Saunders of *Forcipomyia* Meigen (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 109: 530-540.

Grogan Jr., W.L., G.R. Spinelli, R.A. Phillips, and D.L. Woodward. 2004. The male of *Culicoides reevesi* Wirth, with a redescription of the female and new seasonal activity, distribution, and biting records (Diptera: Ceratopogonidae). *Western North American Naturalist* 64: 433-438.

Jamnback, H. 1965. The *Culicoides* of New York state (Diptera: Ceratopogonidae). *New York State Museum Science Service, Albany Bulletin* No. 399. 154 pp.

Jamnback, H. and W.W. Wirth. 1963. The species of *Culicoides* related to *obsoletus* in Eastern North America (Diptera: Ceratopogonidae). *Annals of the Entomological Society of America* 56: 185-198.

Khalaf, K.T. 1954. The speciation of the genus *Culicoides* (Diptera, Heleidae). *Annals of the Entomological Society of America* 47: 34-51.

Linton, Y.L., A.J. Mordue (Luntz), R.H. Cruickshank, R. Meiswinkel, P.S. Mellor, and J.F. Dallas. 2002. Phylogenetic analysis of mitochondrial cytochrome oxidase subunit I gene of five species of the *Culicoides imicola* species complex. *Medical and Veterinary Entomology* 16: 139-146.

Mehlhorn, H., V. Walldorf, S. Klimpel, B. Jahn, F. Jaeger, J. Eschweiler, B. Hoffmann, and M. Beer. 2007. First occurrence of *Culicoides obsoletus*-transmitted bluetongue virus epidemic in Central Europe. *Parasitology Research* 101: 219-228.

Mullen, G.R. 2009. Chapter 12. Biting Midges (Ceratopogonidae). pp. 169-188. *In* Mullen, G.R. and L.A. Durden. Medical and Veterinary Entomology, 2nd Edition. Amsterdam, Elsevier Academic Press.

Page, R.D.M. 1996. Treeview: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358.

Schwenkenbecher, J.M., A.J. Mordue (Luntz), and S.B. Piertney. 2009. Phylogenetic analysis indicates that *Culicoides dewulfi* should not be considered part of the *Culicoides obsoletus* complex. *Bulletin of Entomological Research* 99: 371-375.

Spinelli, G.R. and W.L. Grogan, Jr. 2003. A revision of the Neotropical biting midges of the genus *Paradasyhelea* Ingram and Macfie (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 105: 568-577.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731-2739.

Waugh, W.T. and W.W. Wirth. 1976. A revision of the genus *Dasyhelea* Kieffer of the Eastern United States North of Florida (Diptera Ceratopogonidae). *Annals of the Entomological Society of America* 69: 219-247.

Whitworth, T.L., R.D. Dawson, H. Magalon, and E. Baudry. 2007. DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). *Proceedings of the Royal Society, B* 274: 1731-1739.

Wirth, W.W. 1981. *Paradasyhelea harrisoni* n.sp. from the Auckland Islands, and additional records of subantarctic Ceratopogonidae. *New Zealand Journal of Zoology* 8: 383-386.

Wirth, W.W. and F.S. Blanton. 1969a. New species and records of *Culicoides* from western North America (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 71: 556-567.

Wirth, W.W. and F.S. Blanton. 1969b. North American *Culicoides* of the *pulicaris* group (Diptera: Ceratopogonidae). *Florida Entomologist* 52: 207-243.

Wirth, W.W. and W.L. Grogan, Jr. 1988. The Predaceous Midges of the World. New York, E.J. Brill. 160 pp.

Wirth, W.W. and A.A. Hubert. 1960. Ceratopogonidae (Diptera) reared from cacti, with a review of the copiosus group of *Culicoides. Annals of the Entomological Society of America* 53: 639-658.

Wirth, W.W. and A.P.A. Moraes. 1979. New records and new species of biting midges from salt marshes in California and Mexico (Diptera: Ceratopogonidae). *Pan-Pacific Entomologist* 55: 287-298.

Wirth, W.W. and W.A. Rowley. 1971. A revision of the palmerae group of the genus *Culicoides. Journal of the Kansas Entomological Society* 44: 153-171.

Wirth, W.W. and G.R. Spinelli. 1993. The North American species of the *Forcipomyia* (*Lepidohelea*) *bicolor* subgroup (Diptera: Ceratopogonidae). *Proceedings of the Entomological Society of Washington* 95: 611-634.

Table 4.1. Taxa used in phylogenetic analysis of the subgeneric classification of *Culicoides*.

Taxon	Source	Specimens examined
Forcipomyia	Chan and LeRoux (1971), Wirth and Spinelli (1993), Grogan and Sigrist (2007)	Females, males
Atrichopogon	Boesel (1973)	Females, males
Dasyhelea	Waugh and Wirth (1976)	Females, males
Washingtonhelea frommeri	Wirth and Grogan (1988)	None
Paradasyhelea harrisoni	Wirth (1981)	None
P. ingrami	Spinelli and Grogan (2003)	None
P. macfiei	Spinelli and Grogan (2003)	None
Culicoides		
Amossovia		
C. guttipennis	Blanton and Wirth (1979)	Female, male
C. villosipennis	Blanton and Wirth (1979)	Female, male
Avaritia		
C. chiopterus	Blanton and Wirth (1979)	Female
C. obsoletus	Jamnback (1965)	Female
Beltranmyia		
C. crepuscularis	Blanton and Wirth (1979)	Female
C. hollensis	Blanton and Wirth (1979)	Female, male
Culicoides		
C. cockerellii	Wirth and Blanton (1969b)	Female, male
C. lahontan	Wirth and Blanton (1969b)	Female
Diphaomyia		
C. bergi		Female
C. haematopotus	Blanton and Wirth (1979)	Female
Drymodesmyia		
C. cacticola	Wirth and Hubert (1960)	None
C. copiosus	Wirth and Hubert (1960)	None
C. hinmani	Blanton and Wirth (1979)	Female
Haematomyidium		
C. debilipalpis	Blanton and Wirth (1979)	Female, male
C. paraensis	Blanton and Wirth (1979)	Female
Hoffmania		
C. insignis	Blanton and Wirth (1979)	None
C. venustus	Blanton and Wirth (1979)	Female
Monoculicoides		
C. grandensis	Grogan and Phillips (2008)	None

Table 4.1. *Continued.*

Taxon	Source	Specimens examined
C. variipennis	Blanton and Wirth (1979)	Female, male
Oecacta		
C. furens	Blanton and Wirth (1979)	Female, male
C. stellifer	Blanton and Wirth (1979)	Female, male
Selfia		
Culicoides brookmani	Atchley (1970)	Female
Silvaticulicoides		
C. biguttatus	Blanton and Wirth (1979)	Female, Male
C. spinosus	Blanton and Wirth (1979)	Female
Wirthomyia		
C. stilobezzioides	Jamnback (1965)	Female
Chaetophthalmus group		
C. atchleyi	Wirth and Blanton (1969a)	None
Daedalus group		
C. luglani	Atchley (1967)	None
C. pampoikilus	Atchley (1967)	None
Leoni group		
C. reevesi	Atchley (1967), Grogan et al. (2004)	Female, male
Mohave group		
C. hoguei	Wirth and Moraes (1979)	None
Palmerae group		
C. palmerae	Wirth and Rowley (1971)	Female
C. oregonensis	Wirth and Rowley (1971)	None
Piliferus group		
C. doeringae	Atchley (1967)	Female
C. parapiliferus	Blanton and Wirth (1979)	Female
Stonei group		
C. melleus	Blanton and Wirth (1979)	Female
C. niger	Blanton and Wirth (1979)	Female
Unplaced Species		
C. nanus	Blanton and Wirth (1979)	Female
C. travisi	Blanton and Wirth (1979)	Female

Table 4.2. Character matrix used to infer phylogenetic relationships of Nearctic *Culicoides*. The ancestor group includes *Forcipomyia*, *Atrichopogon*, and *Dasyhelea*.

Taxon	Character																														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Ancestor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Washingtonhelea frommeri	1	1	1	1	?	0	0	0	0	0	0	0	0	0	?	?	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Paradasyhelea harrisoni	1	1	1	1	1	1	0	0	0	1	0	0	0	0	?	?	0	0	3	0	0	0	0	0	0	?	1	0	0	1	0
P. ingrami	1	1	1	1	1	1	?	0	0	1	0	0	0	0	?	?	0	0	3	0	0	0	0	0	0	0	1	0	0	1	0
P. macfiei	1	1	1	1	1	1	?	0	?	1	0	0	0	0	?	?	0	0	3	0	0	0	0	0	0	0	1	0	1	1	0
Culicoides guttipennis	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	2	1	1	0	0	1	0	0	3	0
C. villosipennis	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	2	1	1	0	0	1	0	0	3	0
C. chiopterus	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	0	0	2	0	0	1	1	1	0	1	0	0	0	2	0
C. obsoletus	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	1	0	0	2	0	0	1	1	1	0	1	0	0	0	2	0
C. crepuscularis	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	1	0	0	3	0
C. hollensis	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	1	0	0	2	0
C. cockerellii	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	0	2	0
C. lahontan	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	0	2	0
C. bergi	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	0	1	4	1	1	2	1	1	0	0	1	0	1	1	1
C. haematopotus	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	1	1	4	1	1	2	1	1	0	0	1	0	1	3	1
C. copiosus	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	2	?	?	1	0	1	0	0	2	0
C. cacticola	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1	2	?	?	1	0	1	0	0	3	0
C. hinmani	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	1	2	1	1	0	0	1	0	0	2	0
C. debilipalpis	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	1	0	4	0	0	2	1	1	0	0	1	0	1	1	0
C. paraensis	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	1	0	4	0	0	2	1	1	0	0	1	0	1	1	0
C. insignis	1	1	1	1	1	1	0	0	1	0	1	0	1	0	0	0	0	0	2	0	0	1	1	1	0	1	1	0	0	3	0
C. venustus	1	1	1	1	1	1	0	0	1	0	1	0	1	0	0	0	0	0	2	0	0	1	1	1	0	1	1	0	0	2	0
C. grandensis	1	1	1	1	1	1	1	1	1	2	0	1	0	0	0	0	0	0	3	0	0	2	0	0	0	0	1	1	0	1	0
C. variipennis	1	1	1	1	1	1	1	1	1	2	0	1	0	0	0	0	0	0	3	0	0	2	0	0	0	0	1	1	0	1	0
C. furens	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	1	0	4	0	0	2	1	1	0	0	1	0	1	1	0
C. stellifer	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	1	0	4	0	0	2	1	1	0	0	1	0	1	1	0
C. brookmani	1	1	1	1	1	1	1	0	0	2	1	2	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0	1	0
C. biguttatus	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	2	1	1	0	0	1	0	0	2	0
C. spinosus	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	2	1	0	0	0	1	0	1	2	0
C. stilobezzioides	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	4	0	0	0	1	0	0	0	1	0	1	1	0
C. atchleyi	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	2	0
C. luglani	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	4	0	1	2	1	1	0	0	1	0	1	1	0
C. pampoikilus	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	1	2	1	?	0	0	1	0	0	3	0
C. reevesi	1	1	1	1	1	1	1	2	1	0	0	0	0	0	0	0	1	0	4	0	0	2	1	1	0	0	1	0	1	1	0
C. hoguei	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	1	0	4	0	0	2	1	1	0	0	1	0	1	1	0
C. oregonensis	1	1	1	1	1	1	2	1	1	0	1	0	0	0	0	0	0	0	1	0	0	2	1	1	0	0	1	0	0	3	0
C. palmerae	1	1	1	1	1	1	2	1	1	0	1	0	0	0	0	0	0	0	1	0	1	2	1	1	0	0	1	0	0	3	0
C. parapiliferus	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	0	0	4	1	1	2	1	1	0	0	1	0	1	3	0
C. doeringae	1	1	1	1	1	1	1	2	1	0	1	0	0	0	0	0	0	0	4	1	1	2	1	1	0	0	1	0	1	3	0
C. melleus	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	3	0
C. niger	1	1	1	1	1	1	2	1	1	0	1	0	0	0	0	0	0	0	1	0	0	2	1	0	0	0	1	0	0	3	0
C. nanus	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	2	1	1	0	0	1	0	0	2	0
C. travisi	1	1	1	1	1	1	2	1	1	0	1	0	0	0	0	0	0	0	1	0	0	2	1	1	0	0	1	0	0	3	0

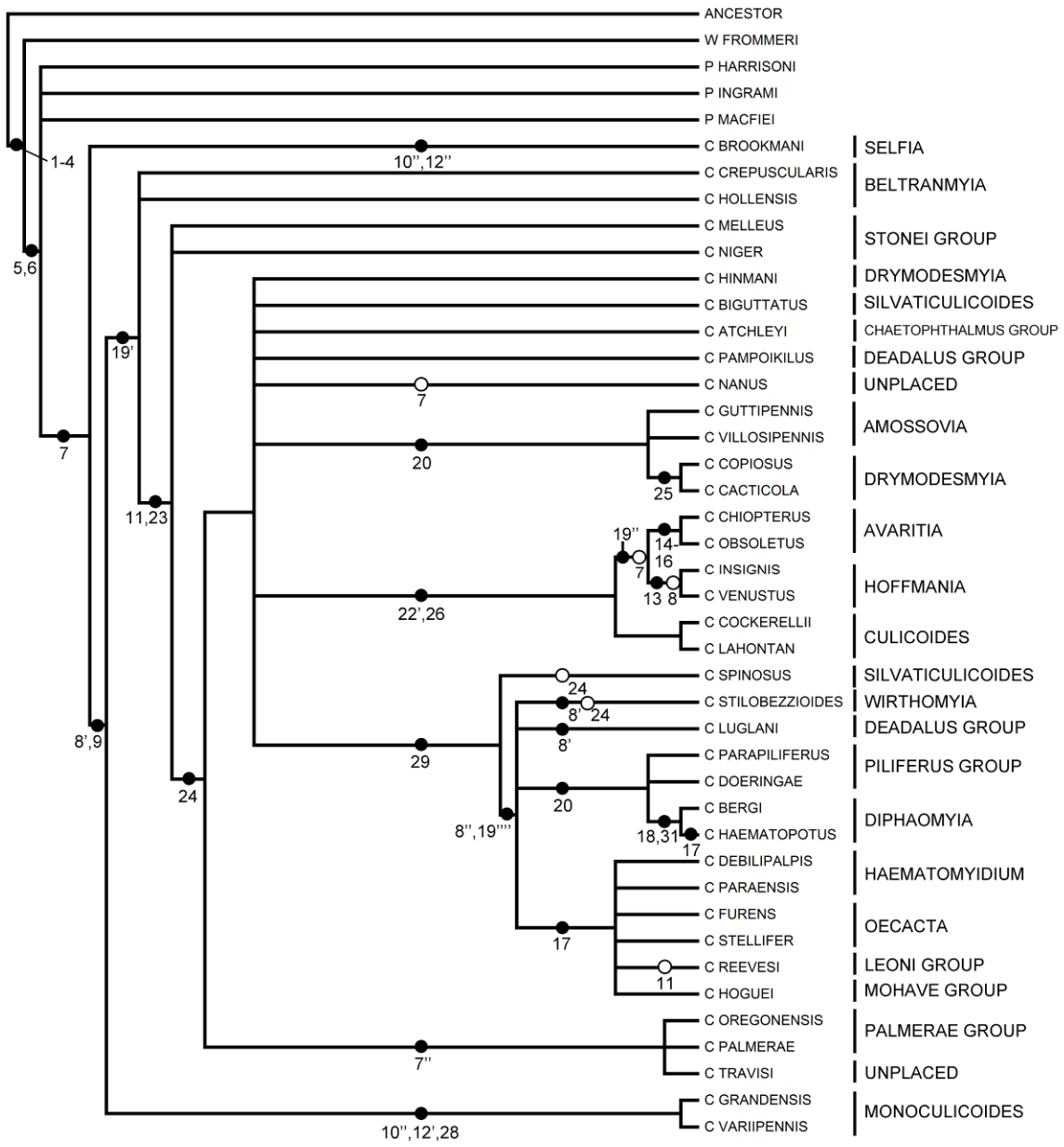


Fig. 4.1. Majority rule (50%) consensus tree for Nearctic *Culicoides* with synapomorphies of clades labeled. The ancestor group is represented by *Forcipomyia, Atrichopogon,* and *Dasyhelea.* Closed circles represent the apomorphic condition, open circles represent the plesiomorphic condition. Characters 21, 27, and 30 are not labeled due to high homoplasy.

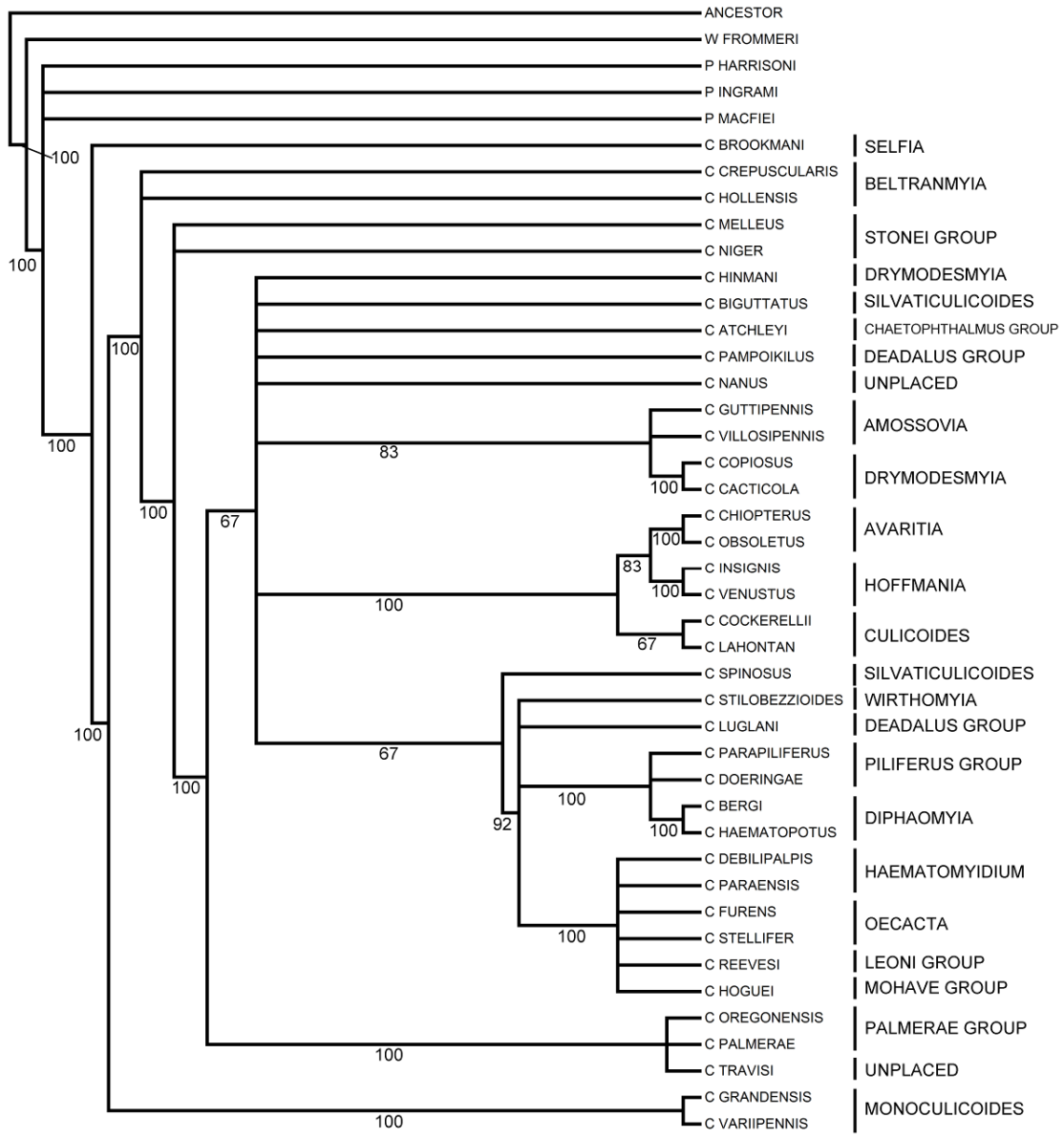


Figure 4.2. Majority rule (50%) consensus tree for Nearctic *Culicoides* of 13 subgenera, 7 species groups, and 2 unplaced species inferred by parsimony analysis from 31 morphological characters. Subgenera and species group names are on right. Numbers represent percentage of trees containing the associated clade. The ancestor taxon includes *Forcipomyia, Atrichopogon,* and *Dasyhelea*.

Table 4.3. Species of *Culicoides* used for maximum likelihood analysis of a fragment of COI.

Taxon	Code	Location	Date
Outgroup			
C. brookmani	jul8	WY: Crook Co.Barlow Canyon	18 Jun 2008
Avaritia			
C. obsoletus	A7	SC: Pickens Co.Clemson Exp. Forest	3 Nov 2008
C. obsoletus	WI18	WI: Juneau Co.Necedah Wildlife Refuge	9 Jun 2009
C. obsoletus	WI23	WI: Juneau Co.Necedah Wildlife Refuge	8 Jun 2009
C. obsoletus	WI24	WI: Juneau Co.Necedah Wildlife Refuge	8 Jun 2009
C. sanguisuga[1]	WI20	WI: Juneau Co.Necedah Wildlife Refuge	10 Jun 2009
Culicoides			
C. cockerellii	jul5	WY: Crook Co.Barlow Canyon	18 Jun 2008
Diphaomyia			
C. baueri[2]	A11	SC: Pickens Co.Clemson Exp. Forest	5 Jun 2008
C. baueri	A88	SC: Charleston Co.: Clemson CREC	30 Apr 2010
C. haematopotus	A1	SC: Pickens Co.: Clemson Exp. Forest	12 Aug 2008
C. haematopotus	A74	SC. Richland Co.: Congaree N.P.	7 Aug 2008
C. haematopotus	A83	SC. Richland Co.: Congaree N.P.	7 Aug 2008
C. haematopotus	CR29	SC: Charleston Co.Clemson CREC	10 Aug 2009
C. haematopotus	WI6	WI: Juneau Co.Necedah Wildlife Refuge	9 Jun 2009
Haematomyidium			
C. debilipalpis	A67	SC. Richland Co.: Congaree N.P.	14 May 2010
C. debilipalpis	A80	SC: McCormick Co.: Hickory Knob S.P.	6 Aug 2009
C. debilipalpis	Jul1	IL: Menard Co.Star Hill Arboretum	19 Jun 2009
C. debilipalpis	Jul6	IL: Menard Co.Star Hill Arboretum	19 Jun 2009
C. paraensis	A76	SC. Richland Co.: Congaree N.P.	7 Aug 2008
Hoffmania			
C. venustus	A19	SC: Pickens Co.: Clemson Exp. Forest	14 Jul 2009
C. venustus	A34	SC: Pickens Co.: Clemson Exp. Forest	14 Jul 09
C. venustus	+	SC: Barnwell Co.33.37N, 81.41S	10 May 2007
Oecacta			
C. furens	A87	SC: Charleston Co.Clemson CR&EC	10 Aug 2009
C. furens	HB5	SC: Georgetown Co.Huntington Beach S.P.	29 Apr 2010
C. stellifer	A6	SC: Pickens Co.: Clemson Exp. Forest	12 Aug 2008
C. stellifer	A31	SC: Pickens Co.: Clemson Exp. Forest	12 Aug 2008
C. stellifer	A39	SC. Richland Co.: Congaree N.P.	7 Aug 2008
C. stellifer	A53	SC. Richland Co.: Congaree N.P.	7 Aug 2008
C. stellifer	A78	SC. Richland Co.: Congaree N.P.	7 Aug 2008
C. stellifer	A90	SC: Charleston Co.: Clemson CREC	1 May 2010

Table 4.3. *Continued.*

Taxon	Code	Location	Date
Silvaticulicoides			
C. spinosus	A9	SC: Pickens Co.: Clemson Exp. Forest	8 May 2007
C. spinosus	A32	SC: Pickens Co.: Clemson Exp. Forest	8 May 2007
C. spinosus	A70	SC. Richland Co.: Congaree N.P.	5 May 2008
C. spinosus	A71	SC. Richland Co.: Congaree N.P.	5 May 2008
C. spinosus	EF3	SC: Pickens Co.Clemson Exp. Forest	22 May 2007
Wirthomyia			
C. stilobezzioides	WI12	WI: Juneau Co.Necedah Wildlife Refuge	8 Jun 2009
C. piliferus group			
C. doeringae	Jul7	CO: Laramie Co.Horsetooth Reservoir	9 Jul 2008
C. parapiliferus	A44	SC. Richland Co.: Congaree N.P.	5 May 2008
C. parapiliferus	WI5	WI: Juneau Co.Necedah Wildlife Refuge	2 Jun 2009
C. parapiliferus	WI19	WI: Juneau Co.Necedah Wildlife Refuge	1 Jun 2009

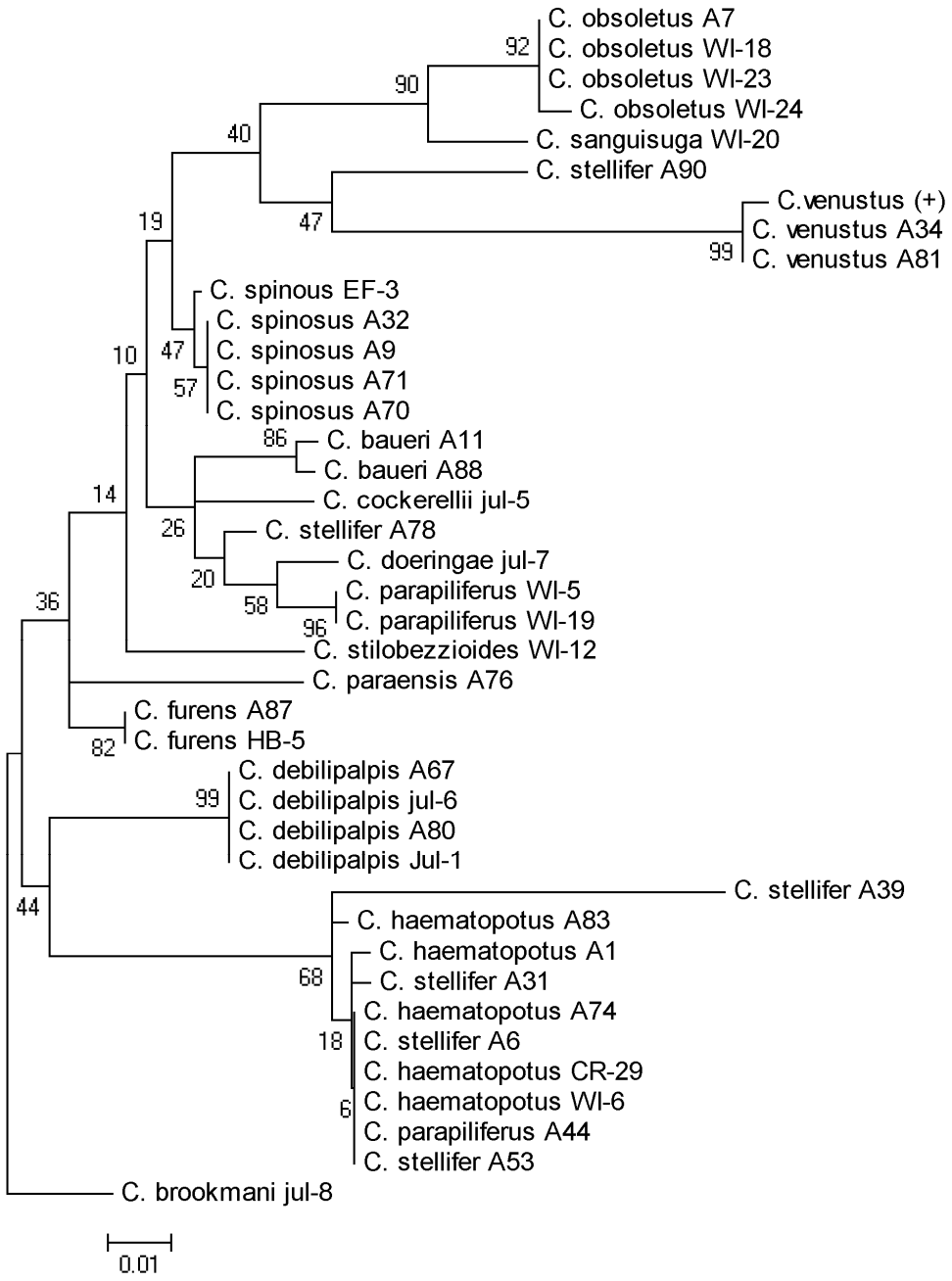


Figure 4.3. Maximum likelihood bootstrap consensus tree of the *Avaritia+Hoffmania+ Culicoides* and the *Diphaomyia+Haematomyidium+Oecacta+Withomyia+C.piliferus* group+*C. leoni* group+*C. mohave* group clades inferred from COI sequences. Numbers indicate bootstrap support. *Culicoides brookmani* was used for the outgroup.

Table 4.4. Sequences of COI for species of *Culicoides* obtained from GenBank used to supplement the analysis the *Avaritia*+ *Hoffmania*+*Culicoides* clade.

Taxon	GenBank Accession No.
Avaritia	
C. bolotinos	AF071928.2
C. bolotinos	AF071929.2
C. bolotinos	AF071930.2
C. bolotinos	AF071931.2
C. chiopterus	AM236748.1
C. chiopterus	AM236749.1
C. chiopterus	AM236750.1
C. chiopterus	AM236751.1
C. dewulfi	AM236706.1
C. dewulfi	AM236707.1
C. imicola	AJ867233.1
C. imicola	AJ867234.1
C. obsoletus	AM236670.1
C. obsoletus	AM236671.1
C. scoticus	AM236650.1
C. scoticus	AM236651.1
C. tuttifrutti	AF069245.2
C. tuttifrutti	AF069246.2
Culicoides	
C. grisescens	AM236731.1
C. grisescens	AM236732.1
C. impunctatus	AM236724.1
C. impunctatus	AM236724.1
C. pulicaris	JF766360.1
C. pulicaris	JF766362.1

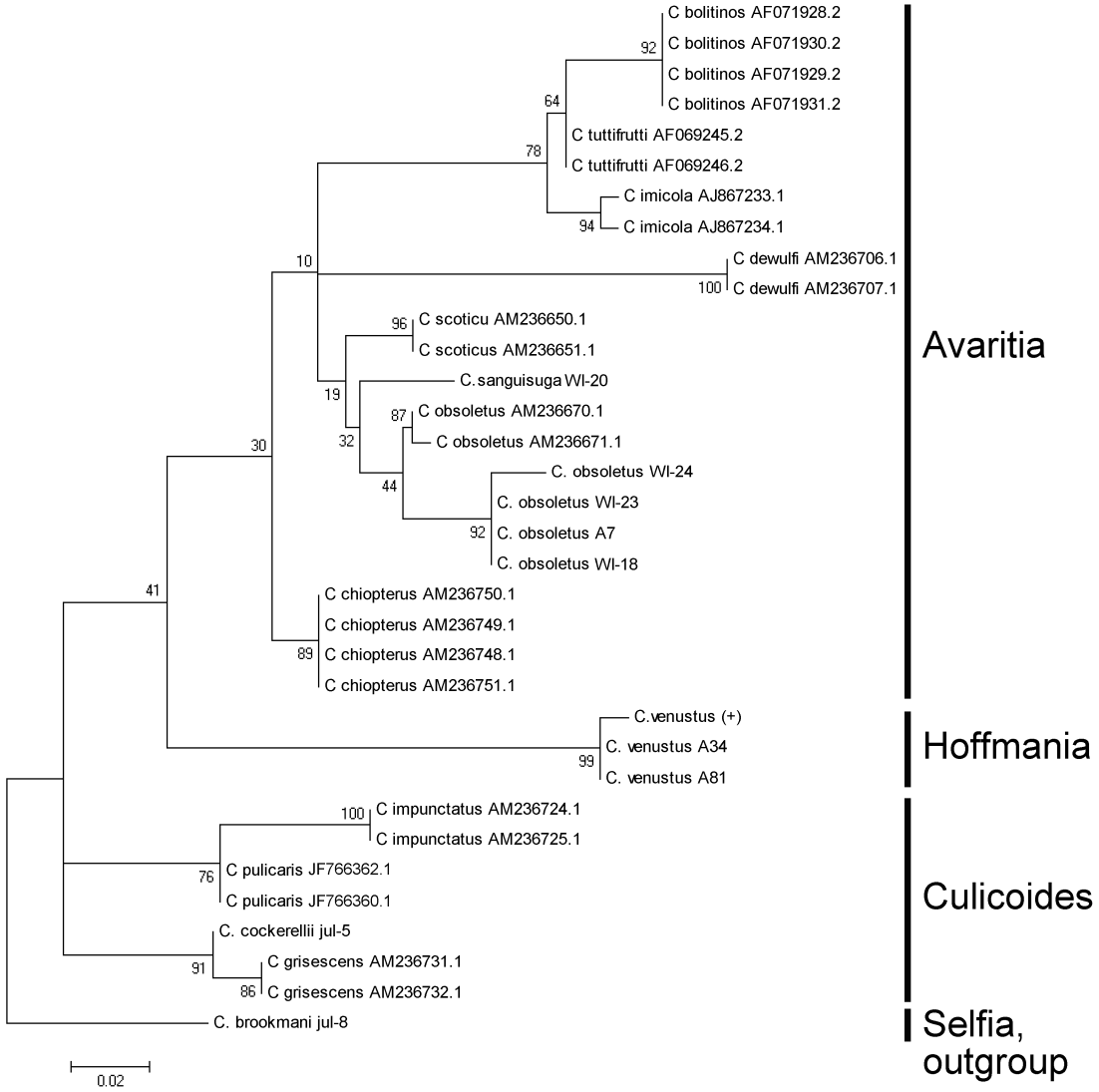


Figure 4.4. Maximum likelihood bootstrap consensus tree of the subgenera *Avaritia, Hoffmania,* and *Culicoides*. Numbers indicate bootstrap support. *Culicoides brookmani* was used for the outgroup.

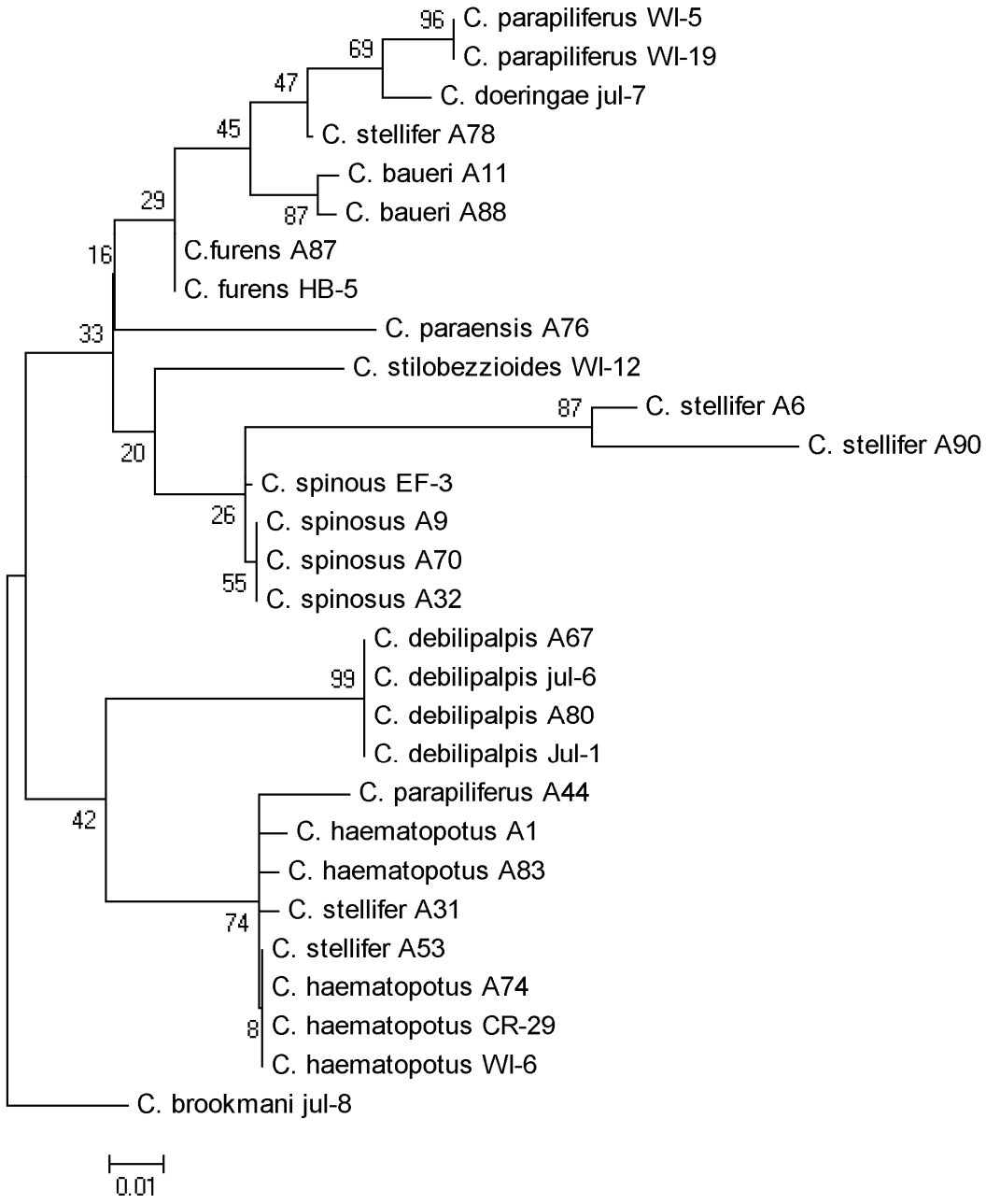


Figure 4.5. Maximum likelihood bootstrap consensus tree of the clade composed of the subgenera *Diphaomyia, Haematomyidium, Oecacta,* and *Wirthomyia*; *C. mohave* group, *C. leoni* group, and *C. piliferus* group; and *C. spinosus*. Numbers indicate bootstrap support. *Culicoides brookmani* was used for the outgroup.

CHAPTER FIVE

ECOLOGY AND PHYLOGENY OF *CULICOIDES*: SYNTHESIS AND FUTURE DIRECTIONS

Much is still unknown about *Culicoides*, especially the ecology and phylogeny. Reciprocally assessing these aspects of *Culicoides* can enhance the study of the genus.

A benefit of understanding the phylogeny of a particular group is the testable hypotheses that can be generated. By examining a phylogeny, the ecology of species with unknown habits can be predicted by examining the habits of closely related species. One example is host associations. If the clade composed of the subgenera *Diphaomyia, Haematomyidium, Oecacta,* and *Wirthomyia* and the associated species groups (Chapter 4) is used as an example, known host associations (Bennet 1960, Blanton and Wirth 1979, Mullens et al. 2006) can be mapped onto the phylogeny (Fig. 5.1). Assuming the relationships among the species reflect the evolutionary divergence, we can predict host associations for taxa with unknown host associations. Because *C. bergi* Cochrane, 1973, and *C. haematopotus* are known to feed on birds, the closely related *C. doeringae* Atchley, 1967, and *C. parapiliferus* can be predicted to feed on birds. *Culicoides hoguei* Wirth and Moraes, 1979, would be predicted to feed on mammals, as all of the most closely related species feed on mammals.

The ecology of an organism can be informative to phylogeny. Ecological data can be used as phylogenetic characters, although they can be difficult to polarize. The hypothesized relationship of the subgenus *Amossovia* to *C. cacticola*+*C. copiosus* of the subgenus *Drymodesmyia* serves as an example (Fig. 5.2). Larvae of the subgenus

Amossovia inhabit tree holes (Blanton and Wirth 1979), whereas larvae of many species of the subgenus *Drymodesmyia* inhabit rotting cacti (Wirth and Hubert 1960). The character state of breeding in tree holes can be used as a synapomorphy to group species of the subgenus *Amossovia,* and the character state of breeding in rotting cacti can be used to group species of the subgenus *Drymodesmyia*. This character would help resolve the polytomy among the species of these two subgenera.

Future Directions

Ecology

Future studies of the ecology of *Culicoides* depend on stable and reliable taxonomy. This is especially true for ecological studies of the immature stages. Techniques to identify larval *Culicoides* are available that do not require rearing specimens to adulthood. Techniques such as DNA barcoding, randomly amplified polymorphisms, restriction fragment length polymorphisms, and DNA hybridization are potential tools to help identify *Culicoides*. These techniques can then be incorporated into a variety of ecological studies. Studies of broad (biogeographic regions) and narrow (individual habitats) scope are needed to gain a full understanding of the ecology of *Culicoides*. For example, genetic variation indicative of species complexes was observed for *C. haematopotus* and *C. stellifer* at a local (state) level. If these species are each a complex of two or more species at a local scale, how many species occur across the entire range of the United States and Canada (Borkent and Grogan 2009)? An ecological study at the biogeographic level likely will reveal more cryptic species and the ecological characters could help delimit the species, as in the *C. variipennis* complex (Holbrook et

al. 2000, Schmidtmann et al. 2011). Ecological studies at the local scale (habitat level) can help inform larger scale studies by providing the specific habitat of a species and ecological parameters indicating where in that habitat a species is likely to occur.

Phylogeny

One of the major difficulties facing the study of *Culicoides* is taxonomic instability. A lack of cladistic analysis at subgenus and genus levels and researchers examining only local faunas to create classification systems have created taxonomic chaos in the genus. A global cladistics analysis of the genus can alleviate the chaos by basing subgeneric classifications on synapomorphies. Such a system would allow researchers in any region to place new species into correct subgenera and species groups.

The phylogenies presented throughout this dissertation were based largely on characters from adults. The inclusion of characters from eggs, larvae, and pupae can provide greater resolution to the resulting phylogenies. The shape of the eggs is a synapomorphy of the genus *Dasyhelea* (Borkent 1995). The seta arising from cuticular projections is a synapomorphy of the subfamily Forcipomyiinae (Borkent 1995). In the *Culicoides*, members of the subgenus *Monoculicoides* have a heavily sclerotized epipharynx, whereas other species of the genus have a less sclerotized epipharynx (Kettle and Lawson 1952). This character could serve as a synapomorphy for the subgenus. Molecular data based on multiple genes can be used to assess morphological phylogenies. Much is still to be learned about the phylogeny of Ceratopogonidae and respective taxa, as evidenced by the discovery of a new synapomorphy (scutal areolae) for the family in one of the most thoroughly studied suborders of Diptera.

Closing Remarks

Too often scientists become so involved in their own areas of expertise that they develop tunnel vision and ignore other areas of biology or science that might help enlighten a situation. Such an example was seen in this study where ecological analyses failed to converge on a predictive model for a frequently collected species (*C. haematopotus* examined in Chapter 2) and phylogenetic analysis demonstrated that there might be more than one species. If we can step outside our area of expertise and use different methodologies to approach a problem, we can enhance our respective fields.

References

Bennet, G.F. 1960. On some ornithophilic blood-sucking Diptera in Algonquin Park, Ontario, Canada. *Canadian Journal of Zoology* 38: 377-389.

Blanton, F.S. and W.W. Wirth. 1979. The sand flies (Culicoides) of Florida (Diptera: Ceratopogonidae). Arthropods of Florida and Neighboring Land Areas 10. 204 pp.

Borkent, A. 1995. Biting midges in the Cretaceous amber of North America (Diptera: Ceratopogonidae). Leiden: Backhuys, 237 pp.

Holbrook, F.R., W.J. Tabachnick, E.T. Schmidtmann, C.N. McKinnon, R.J. Bobian, and W.L. Grogan, Jr. 2000. Sympatry in the *Culicoides variipennis* complex: a taxonomic reassessment. *Journal of Medical Entomology* 37: 65-76.

Kettle, D.S. and J.W.H. Lawson. 1952. The early stages of British biting midges *Culicoides* Latreille (Diptera: Ceratopogonidae) and allied genera. *Bulletin of Entomological Research* 43: 421-467, XIX plates.

Mullens, B.A., C.J. Cardona, L. McClellan, C.E. Szijj, and J.P. Owen. 2006. *Culicoides bottimeri* as a vector of *Haemoproteus lophortyx* to quail in California, USA. *Veterinary Parasitology* 140: 35-43.

Schmidtmann, E.T., M.V. Herrero, A.L. Green, D.A. Dargatz, J.M. Rodriquez, and T.E. Walton. 2011. Distribution of *Culicoides sonorensis* (Diptera: Ceratopogonide) in Nebraska, South Dakota, and North Dakota: Clarifying the epidemiology of bluetongue disease in the northern great plains region of the United States. *Journal of Medical Entomology* 48: 634-643.

Wirth, W.W. and A.A. Hubert. 1960. Ceratopogonidae (Diptera) reared from cacti, with a review of the copiosus group of *Culicoides*. *Annals of the Entomological Society of America* 53: 639-658.

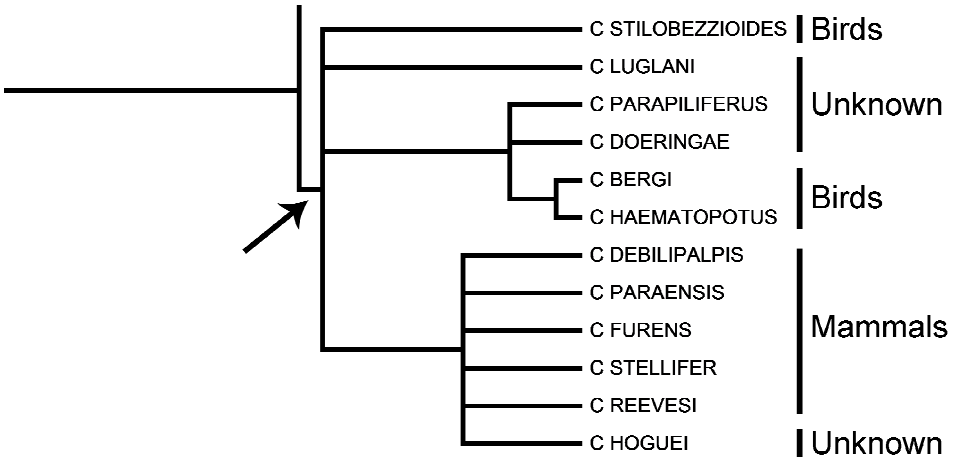


Figure 5.1. Known host associations mapped on the phylogeny of *Culicoides* of the *Diphaomyia, Haematomyidium, Oecacta,* and *Wirthomyia* subgeneric clade inferred in Chapter 4. Arrow denotes the referenced clade.

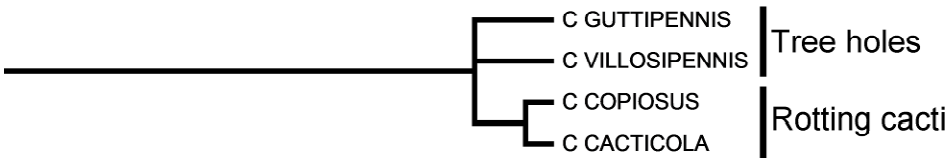


Figure 5.2. Larval habitats of four species of *Culicoides* mapped onto a phylogeny of those species.