A STUDY OF THE TAXONOMY, ECOLOGY, AND SYSTEMATICS OF *CULICOIDES* SPECIES (DIPTERA: CERATOPOGONIDAE) INCLUDING THOSE ASSOCIATED WITH DEER BREEDING FACILITIES IN SOUTHEAST TEXAS

A Thesis

by

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ABSTRACT

The biting females of a number of species of *Culicoides* Latreille have a great economic impact throughout the globe. They act as vectors of various disease pathogens, such as Bluetongue, Epizootic Hemorrhagic, African Horse Sickness, various protozoa and nematodes, and in some areas occur in such huge numbers that outdoor activities for humans and domestic animals are severely limited. In spite of their great impact, there are still numerous fundamental aspects of this group that remain unknown. This study fills some of these gaps of knowledge for this genus, including some species of *Culicoides* in Texas.

In this study, the pupal stage of a vector of the disease-causing agent of Bluetongue in North America, *Culicoides sonorensis* Wirth and Jones, is described in detail for the first time. A multitude of approaches were used in this description to offer one of the most in depth comparative morphological studies of any ceratopogonid pupa.

This study also interpreted the locality and seasonality data of several *Culicoides* species by surveying their association with deer breeding facilities in southeastern Texas. A total of twelve species were collected at two sites in the Brazos Valley, with one species being newly recorded in Texas. A synopsis is provided for each species, including seasonality, distribution, feeding habits, and larval habitats.

Molecular study provided 658 base pair CO1 sequences from eight species collected in this study as well as from an additional five species collected in Canada, England, and Ireland; seven of these sequences are new to GenBank. Using a

morphological character matrix, a strict consensus tree from 56 equally parsimonious trees was constructed. Analysis of this tree provides evidence for the monophyly of the subgenus *C.* (*Monoculicoides* Khalaf) as well as five synapomorphies for the group. Further phylogenetic resolution of some species within the subgenus is also provided. The analysis also indicates that *C. rarus* Das Gupta has been misplaced in this subgenus.

DEDICATION

I dedicate this thesis first and foremost to my wife Arli Shults. Thank you for your constant love, support, guidance, and grammatical expertise. You have been a wonderful source of joy and have helped me through many challenges. I can't even begin to express my appreciation for all that you've done for me. I'm just grateful for all the time that we've had together, and for all the time we still have.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Vector Biology

Species of *Culicoides* Latreille are small members of the family Ceratopogonidae (Order: Diptera). Both male and female *Culicoides* feed on nectar to fuel their flight. Only the females take a blood meal from a vertebrate host, using the protein-rich source to develop their eggs. The majority of *Culicoides* species feed on birds, lizards, and mammals (including humans), with one subgenus feeding on blood-fed flies. Approximately seven to ten days after feeding, eggs are laid on a moist substrate such as animal feces, detritus, or on the muddy margins near lakes, ponds, pools, or streams (Jamnback 1965). The immature stages of *Culicoides* can be found in a variety of different habitats, ranging from damp vegetation and manure to very wet or fully aquatic microhabitats (Borkent 2014). The larva feed on microorganisms and small invertebrates. *Culicoides* pupae are mobile, though normally slow and sessile, and usually rest near the surface of wet substrate in order to breathe (Borkent 2014). Adult emergence varies from species to species, though most are active from late spring to early fall. However, as this study shows, some species can even be collected as early as February in southeastern Texas.

Some species of *Culicoides* are of enormous economic importance as vectors of many disease pathogens affecting livestock worldwide, including at least 66 viruses, 15 protozoa and 23 nematodes (Borkent 2004). These include the transmission of the

disease agents of Bluetongue (BT), African Horse Sickness, Bovine Ephemeral Fever, Akabane Virus, and Epizootic Hemorrhagic Disease (EHD) (Holbrook 1996). These pathogens may be fatal to the infected animal. Of particular importance in North America are EHD and BT. While the diseases are different, the clinical signs are the same (Mellor et al. 2000). These diseases adversely affect domestic and wild ruminants, such as cows, sheep, deer, and horses. Symptoms of infections include swelling of the tongue, internal hemorrhaging, permanent lameness, spontaneous abortions, congenital deformities and death (Holbrook 1996; Howarth et al. 2001; Maclachlan and Gard 2009; Schmidtmann 2011). The principle vector of BT virus in North America is *Culicoides sonorensis* Wirth and Jones, and the principle vector of EHD virus is *Culicoides variipennis* (Coquillett) (Borkent 2014). As the deer breeding industry continues to grow at a rapid rate (Anderson et al. 2007), the spread of disease through deer facilities could continue to be a problem.

Taxonomy of Culicoides

Adult *Culicoides* can be morphologically distinguished from other members of Ceratopogonidae by the lack of palisade setae, tarsal claws of equal length, and the lack of a terminal nipple on the apical flagellomere. Some also have distinctively patterned wings, which aid in determination of species (Wirth et al. 1985). Most of the diagnostic characters, however, cannot be seen using only a dissecting microscope. Specimens need to be properly cleared, slide-mounted, and viewed through a compound microscope for confident identification.

There are 1,355 species of *Culicoides* worldwide (Borkent 2015), with 100 species placed in 13 subgenera in North America. However, there are 53 other species that are either separated into poorly defined species groups or not placed at all (Borkent and Grogan 2009). Of the *Culicoides* species that have been described worldwide, only 13% are known as larvae and 17% as pupae (Borkent 2014). Remarkably, until this study, the pupae of *C. sonorensis* had never formally been described. This species has been kept in laboratory colonies for almost 60 years, and yet the genome for *C. sonorensis* was available before a pupal description. Scattered and missing information is common within *Culicoides* literature and can pose quite a challenge.

There are very successful control strategies that target the immature stages of blood-feeding, pathogen-carrying flies (e.g. Culicidae, Simuliidae). It is puzzling why such a significant portion of fundamental taxonomic work has not been completed, for at the very least those species of *Culicoides* involved in pathogen transmission. Control of pests depends upon a thorough basic knowledge of the genetics, systematics, taxonomy, morphology, biology, ecology, and the geographical and seasonal distribution of those various species (Blanton and Wirth 1979).

Texas Culicoides

Currently, there are 40 *Culicoides* species listed in Texas (Fox 1955, Borkent and Grogan 2009); however, much of the state has yet to be sampled and there are no keys available. Fortunately, keys are available for New Mexico (Atchley 1967), Oklahoma (Khalaf 1957) and Florida (Blanton and Wirth 1979), with the latter including both

adults and pupae and maps with the broader distribution of each species. These keys allowed for the identification of material studied here. There have been some useful and extensive surveys carried out in limited areas within Texas. Wirth (1955) sampled *Culicoides* at the Texas Agricultural Experiment Station in Sonora and collected and described three new species from there (*C. peconensis* Wirth, *C. neopulicaris* Wirth, *C. bottimeri* Wirth). Wirth and Hubert (1960), in collaboration with Robert Jones, sampled *Culicoides* using light traps and reared a long series of specimens from cacti from California, Arizona, and Texas. Their paper described six new species, added new records of species occurring in Texas, and included a key to the species of the *copiosus* group. Jones (1961) completed a large survey of possible breeding sites for *Culicoides* in seven counties in Texas and added valuable information on the breeding sites for many species in the state. Wirth and Jones (1957) and Holbrook et al. (2000) surveyed populations of *Culicoides* throughout Texas, and found all three species (then considered subspecies) of the *variipennis* complex. Most recently, Vigil et al. (2014) identified new records of species of *Culicoides* present in the southeastern United States, including those of potential BTV and EHDV vectors and in Texas.

Phylogenetic Analysis of Culicoides

Phylogenies based on rigorous methods offer a means to infer evolutionary histories, relatedness among species, and provide a framework to study shared behaviors or biological adaptations within clades. This study is the first morphologically based cladistic analysis of any group of species of *Culicoides*, including those of the subgenus

of *C.* (*Monoculicoides* Khalaf). Previous authors have proposed various relationships between species of *Culicoides* using molecular methods (for a comprehensive list see Table 3 in Harrup et al. 2014). Gomulski et al. (2005) constructed a phylogeny of species of the subgenus *C.* (*Avaritia* Fox) using ITS2 sequences. Perrin et al. (2006) constructed a phylogeny of the *Culicoides* species of France based on nuclear ITS_1-rDNA sequences. The latter authors included four species of *C.* (*Monoculicoides*) in their study: three from France and one from North America. The study provided evidence to the genetic similarity within the subgenus. Two methods for analyzing *C.* (*Monoculicoides*) were used in this study. One method through the interpretation of COI sequences, testing the ability to barcode, and the other used a morphological character matrix to conduct a phylogenetic analysis.

CHAPTER II

FORMAL DESCRIPTION OF THE PUPA OF *CULICOIDES SONORENSIS*

Introduction

The Nearctic species *Culicoides sonorensis* Wirth and Jones and *Culicoides variipennis* (Coquillett) are the principle vectors of viruses associated with both Bluetongue and Epizootic Hemorrhagic Disease in North America (Jones et al. 1983, Tabchnick 1996, Gerry et al. 2001, Rudder et al. 2012). *Culicoides sonorensis* was first described (in the adult stage only) as a subspecies of *C. variipennis* by Wirth and Jones (1957). It was given full species status by Holbrook et al. (2000), who used allele frequency data and a few minute morphological differences to distinguish three species within the *variipennis* complex, namely *C. variipennis*, *C. occidentalis* Wirth and Jones, and *C. sonorensis*. However, considering its recent species-level status, a definitive pupal description of this species has never been published, other than the partial description of the pupa by Borkent 2012 and 2014. Wirth (1952) may have examined, in part, the larva and pupa of *C. sonorensis* as part of his brief description of these stages (as *C. variipennis*) in California, but this is uncertain. Murphree and Mullen (1991) described the larvae of *C. variipennis* and *C. occidentalis* (as subspecies of *C. variipennis*). Abubekerov 2014 described the egg and all larval instars of *C. sonorensis*. The pupa of *C. variipennis* has been briefly described by several authors (Malloch 1915; Thomsen 1937; Fox 1942; Jones 1955; Jamnback 1965; Blanton & Wirth, 1979; Weber, 2001), however, the pupa of *C. occidentalis* is undescribed.

The morphological differences between the adults of these three species are subtle and often difficult to discern. The female of *C. variipennis* can be identified based on shape of the third palpal segment, but those of *C. sonorensis* and *C. occidentalis* cannot be separated. The males of the three species are distinguished by small differences in the density of spicules on the aedeagal membrane and varying degrees of wing pigmentation. These minute differences between species can be difficult to interpret, which leads to uncertainties about correct identification. These uncertainties indicate the need for further study of the adults as well as the undescribed or poorly known immatures. Studies of the pupae of other species of *Culicoides* strongly indicate that there are good morphological differences between them, once understood (e.g. Jamnback 1965; Kettle and Lawson 1952; Nevill 2007 and 2009). It is imperative that further morphological studies be undertaken to better understand each stage. In this chapter is a description of the pupa of *C. sonorensis*, providing a platform for a better understanding of this stage in the *variipennis* complex and other species of *Culicoides*. This is not only the first detailed pupal description of *Culicoides sonorensis*, but also the most in-depth pupal description for any species of *Culicoides* to date. Studying the immature stages may prove to be crucial in understanding the identity of these species, as seen in this study.

One novel contribution here was the use of scanning electron microscope (SEM) photography to thoroughly and systematically photograph each region and important structure of a Ceratopogonidae pupa. Even with the highest quality specimens, there are major limitations to what can be seen using compound and dissecting microscopes. The

use of an SEM allowed for a definitive view of pupal structures. Analyses of these photos provided insight into both form and function.

Materials and Methods

Pupae of *Culicoides sonorensis* were obtained from the "AK" colony (originally collected from Owyhee County, Idaho) at the Arthropod-Borne Animal Disease Research Unit in Manhattan, Kansas, and the Van Ryn (VR) colony (originally collected from San Bernardino County, California) at the University of California Riverside in Riverside, California. Pupae of *Culicoides nubeculosus* (Meigen) were obtained from a colony maintained at the Pirbright Institute in the United Kingdom. Slide mounted exuviae of various species of the subgenus *C.* (*Monoculicoides* Khalaf) were borrowed from the Canadian National Collection and the United States National Museum. Exuviae of *C. occidentalis* and *C. riethi* Kieffer were collected from White Lake, 5 km SW Okanagan Falls, British Columbia, Canada (49°18'27.51"N 119°138'00.23"W, 4-V-2014, A. Borkent).

Whole pupae and exuviae were studied in alcohol and glycerin using a Leica S6D dissecting microscope. Pupal exuviae were slide-mounted in Canada balsam, following the technique of Borkent & Spinelli (2007), and observed under a Nikon Alphashot-2 YS2 compound microscope. Measurements and statistics were reported: range (mean, standard deviation (SD), total number measured (n)). Statistical tests and analyses were done using the software program IBM SPSS statistics version 21. Illustrations were drawn using a Zeiss camera lucida mounted on a Zeiss Standard 16

compound microscope with DIC objectives and condenser. Several pupae of *C. sonorensis* were critical point dried, sputter-coated with gold, and examined at the Microscopy and Imaging center at Texas A&M University with a Scanning Electron Microscope (model Jeol 6400). Voucher specimens of *C. sonorensis* were deposited in the insect collection at Texas A&M University.

Results

The organization of the following description follows, in general, the generic descriptions by Borkent (2014). Features present in all *Culicoides* are not repeated here. The presence of D-1, V-1 or V-2 is not reported from any of the abdominal segments because these could not be viewed in the SEM specimens due to longitudinal compression of the abdominal segments (these sensilla were covered by the preceding segment). These minute sensilla are challenging to see in slide mounted material.

Culicoides sonorensis

Only *Culicoides* pupa with the dorsal apotome densely covered in spines of varying sizes, with larger spines more ventrally, smaller spines dorsally, and spines on the inner margin of the DA-1-H tubercle (Figs. A-2A, A-2B, and A-8C), dorsal apotome without dorsal longitudinal ridges, the very apical margin of the thoracic pedicel with dark pigmentation (Fig. A-3A), apical 0.2-0.3 of the respiratory organ with dark pigmentation, contrasting with lighter brown more basally (Fig. A-13B), 2-3 pores on the midlength portion of the respiratory organ (Figs. A-3A, A-13A, and A-13B), D-1-T

and D-2-T longer than D-5-T (Figs. A-10 and A-11B), L-2, L-3, and L-4 of abdominal segments 4-7 each on a bifid tubercle with elongate and slender apices (Fig. A-35), segment 8 with seven sensilla (Figs. A-27B and A-28), anterior portion of segment 9 not greatly swollen (Figs. A-30 and A-31), tip of the terminal process dark (Fig. A-31).

Male (Figs. A-6B and A-7B) habitus as in figures 1, 6 and 7. Total length = 1.68-2.25 (2.05, 0.173 SD, n=12) mm. Light brown coloration throughout. **Head:** Dorsal apotome (DA) (Figs. A-2A and A-8C), covered in short spines, without dorsomedial tubercle, dorsal margin rounded, lateral margins with distinct points, DA length = 0.26-0.3 (0.28, 0.011 SD, n=12) mm; DA width = 0.175-0.195 (0.185, 0.007 SD, n=12) mm; DAW/DAL= 0.621-0.679 (0.657, 0.02 SD, n=12) mm. DA-1-H medium long, thick seta on well-developed tubercle, DA-2-H dorsal to tubercle base, medial portion of tubercle with short spines, DA-1-H apex not extending beyond ventral margin of DA. Dorsal cephalic sclerite (Figs. A-3B, A-8A, A-8B, and A-11A) with 1 long, 1 short seta. Palpus (Figs. A-2C and A-9) extending posteriorly to posterolateral margin of labium, CL-1-H about twice length of CL-2-H, O-1-H twice length of O-3-H; O-2-H, O-4-H separated medially by O-1-H and O-3-H. **Thorax:** Cephalothorax length = 0.94-1.05 (0.98, 0.042 SD, n =12) mm. Pedicel of respiratory organ short with dark banding at apex. Respiratory organ (RO) (Figs. A-3A, and A-13) elongate, slender, apex dark, smooth, mid-length portion with scales, without annulations, with short membranous base, dark banding on posterior margin, with open cross-shaped pores closely abutting in single row at apex, 2-3 closed subbasal pores, RO length = 0.28-0.35 (0.313, 0.019 SD, n=12) mm; RO width = 0.028-0.030 (0.029, 0.001 SD, n=12) mm; ROW/ROL = 0.086-0.107

(0.094, 0.006 SD, n=12) mm, tracheal tube slightly curved along length distally, with reticulations. Anterolateral sensilla one long, one short on well-developed tubercle (Fig. A-12). Mesonotum (Figs. A-3B, A-10, A-11B, and A-12A) with small bumps anterior to D-5-T, smooth posteriorly, D-1-T, D-2-T short, stout, each on elongate, rounded tubercle, D-3-T posterior to small rounded tubercle bearing long, slender D-4-T, D-5-T miniscule, on small rounded tubercle, D-1-T, D-2-T, D-5-T in longitudinal row. Wing with apical tubercle (Fig. A-21A); halter apex, hind leg slightly separated (Fig. A-18B). Metathorax completely separated medially (Fig. A-10A) with long, thin M-3-T near anterior margin, M-1-T, M-2-T more posterior (Figs. A-3C, A-14, A-15, and A-16A).

Abdomen: Tergite 2-7 each with darker pigmentation as medial group of three patches, with anterolateral pair, sternites 3-7 each with two medial patches, with anterolateral pair (Fig. A-32). Tergite 1 (Figs. A-4A, A-14, A-15, A-16B, and A-17) with long D-3-I, short D-2-I, D-7-I anterior, campaniform D-4-I, short D-8-I, and long, thin D-9-I on short tubercle posteriorly, L-2-I, L-3-I short separated medially by long, thin L-1-I on lateral margin (Fig 17B); segments 2, 3 sub-equal in length, shagreen on posterolateral margin. Chaetotaxy, shagreen of tergite 2 (Fig. A-18) similar to tergite 4, without elongate tubercles, tiny L-2-II, L-4-II separated medially by long, thin L-3-II on anterolateral margin. Chaetotaxy, shagreen of segment 3(Figs. A-19, and A-21A) similar to that of segment 4. Tergite 4 (Figs. A-4B and A-20-22) with short D-2-IV on short, pointed tubercle, thin D-3-IV on elongate, pointed tubercle, D-5-IV, D-4-IV, D-7-IV, D-8-IV, D-9-IV in transverse row, arranged medially to laterally, tiny D-5-IV on slightly formed tubercle, D-4-IV on small rounded tubercle, D-7-IV on small pointed tubercle,

D-8-IV, D-9-IV each long on elongate pointed tubercle, D-8-IV seta thicker than D-9-IV; L-1-IV short seta on small rounded tubercle, L-2-IV, L-4-IV short setae, each on elongate pointed tubercle, separated by short, thin L-3-IV on elongate tubercle; sternite 4 with tiny V-5-IV on small rounded tubercle, V-6-IV thin on pointed tubercle, small V-7-IV on elongate pointed tubercle, ventral setae in transverse row, shagreen along anterior, posterior margins, lateral portion with scattered shagreen in area dorsal, ventral to L-1-IV. Segments 5 and 6 (Figs. A-23-25) with similar chaetotaxy, shagreen to that of segment 4. Segment 7 (Figs. A-26 and A-27A) with similar chaetotaxy to segment 4, without lateral shagreen. Segment 8 (Figs. A-27B and A-28) chaetotaxy with only seven sensilla: D-8-VIII, D-9-VIII, L-2-VIII, L-3-VIII, L-4-VIII, V-6-VIII, V-7-VII, without lateral shagreen, L-3-VII on bifid tubercle, apices also bifid (Fig 28A). Segment 9 (Figs. A-5 and A-29-31) without lateral shagreen, not strongly modified, genital lobes extending to posterior margin, terminal processes closely approximated basally, each projecting posterodorsolaterally, tapering to pointed, dark apex.

Female (Figs. A-6A and A-7A) similar to male other than sexual differences on segment 9 (Figs. A-5 and A-31) and the following: total length = 1.81-2.22 (2.05, 0.148 SD, n=12) mm, ventral margin of the DA not extending past the DA-1-H apex, DA length = 0.23-0.25 (0.24, 0.006 SD, n=12) mm, DA width = 0.18-0.21 (0.2, 0.008 SD, n=12) mm, DAW/DAL = 0.76-0.89 (0.84, 0.036 SD, n=12) mm, Cephalothorax length = 0.97-1.07 (1.01, 0.038 SD, n=12) mm, RO length = 0.30-0.35 (0.32, 0.016 SD, n=12) mm, RO width = 0.028-0.030 (0.029, 0.001 SD, n=12) mm, ROW/ROL = 0.086-0.100 (0.09, 0.005 SD, n=12) mm. (Fig. A-33)

Discussion

A continuing challenge in the systematics of the genus *Culicoides* is the lack of adequate diagnoses for many of the subgenera and species groups currently recognized; the existing diagnoses being almost entirely based on structures of adult females (Borkent 2015). The interpretation of characters of the immatures is markedly deficient. Indeed, it has only been recently that pupae of the genus could be recognized as such (Borkent 2014). It is vital to this discussion to note that the pupae of species of the subgenus *C.* (*Monoculicoides*) are the only group of *Culicoides* with the apical 0.2-0.4 of the respiratory organ dark brown, contrasting with the light brown pigmentation more basally (in some with dark pigmentation at very base) and setae L-2, L-3 and L-4 on each of segments 3-8 each on a bifid tubercle. These features were provided by Kettle and Lawson (1952), who described and keyed the British fauna, with the additional character that the tips of the bifid tubercles of the lateral abdominal setae were darkly pigmented (they referred to these as "two large, dark spines"). The darker apical pigmentation of the tubercles appears in many, but not all, species of *C.* (*Monoculicoides*). Additionally, there appears to be some intraspecific variation in the amount of pigmentation, and further research is needed as to its significance.

The species of *C.* (*Monoculicoides*) in the Nearctic have all been reared from at least the pupal stage. The diagnosis of the pupa of *C. sonorensis* in the results section separates this species from those of all other *Culicoides*. The pupa of *C. sonorensis* and *C. variipennis* are similar but can be distinguished by the shape of the tubercles bearing L-2, L-3, and L-4 on abdominal segments 4-7. In *C. sonorensis*, the tips of the tubercle

are much longer and more pointed, whereas in *C. variipennis*, they are shorter and more blunt. (Fig. A-35) The pupa of *C. sonorensis* may be separated from those of *C. occidentalis* and *C. grandensis* Grogan and Phillips 2008 on the basis of the pattern of cuticular spines on the dorsal apotome. In *C. occidentalis*, short spines cover roughly 75% of the dorsal apotome; in *C. sonorensis*, nearly 90% is covered in spines of various sizes (Fig. A-34). *C.* grandensis lack these spines all together. The pupa of *C. sonorensis*, with dorsal sensilla D-1-T and D-2-T longer than D-5-T (Fig. A-10), may be separated from those of *C. shemanchuki* Grogan and Lysyk 2015, wherein all three are of near equal size. The pupa of *C. sonorensis* differs from *C. riethi* in the shape of abdominal segment 9. In *C. riethi*, the anterior portion of the 9th segment is swollen and the posterior portion strongly truncated. In *C. sonorensis*, the lateral margins of abdominal segment 9 are uniformly straight to slightly truncated, with little to no swelling anteriorly.

Kettle and Lawson (1952) provided a key to the pupae of *C.* (*Monoculicoides*) of Britain (as the *nubeculosus* group) including *C. nubeculosus*, *C. stigma* (Meigen), *C. parroti* Kieffer, and *C. riethi* [they did not include *C. puncticollis* (Becker), described by Dzhafarov (1964)]. There are otherwise only two further *C.* (*Monoculicoides*) species in Europe, *C. helveticus* Callot, Kremer and Deduit, briefly described by Glukhova (1989) and *C. longicollis* Glukhova, not known in the pupal stage. The only other species of *C.* (*Monoculicoides*) described as pupae are *C. homotomus* (Jeu and Rong, 1974, 1981) and *C. cornutus* (De Meillon, 1937).

The pupa of *C. sonorensis* may be separated from those of *C. nubeculosus* by the dorsal apotome. The dorsal portion of the dorsal apotome of *C. nubeculosus* has a low, shallow tubercle from which 2-4 dorsal-ventral ridges extend to the dorsal margin of the dorsal apotome (Fig. A-34), whereas the dorsal apotome of *C. sonorensis* lacks this character.

Borkent (2014) provided a diagnosis and description of *Culicoides* at the generic level. This study indicates the need for an amendment of that diagnosis. The ocular setae are notoriously difficult to see (Fig. A-9) and Borkent (2014) reported the presence of only one campaniform sensillum. The best way to view these setae is to remove the dorsal apotome and "face" in one piece. With this method, we were able to see the area more clearly and discovered that *C. sonorensis* has two ocular campaniform sensilla. Though perhaps not applicable to species identification, this could be a possible subgeneric synapomorphy, and should be re-examined throughout the genus in more detail.

Male and female *Culicoides* pupae can be distinguished by the presence or absence of genital lobes on the ventral side of the 9th segment, respectively (Figs 30 and 31). There is a second sexual dimorphism for *C. sonorensis* based on the size of the dorsal apotome (DA). The width of the DA overlaps between the sexes, though the females tend to be slightly wider. The males have a longer DA, and when slide mounted correctly, the male DA-1-H does not extend past the posterior margin of the DA. This is unlike females, in which the seta does extend posteriorly beyond the posterior margin (Fig. A-2A and B). This difference is most clearly seen in the ratio of dorsal apotome

width to length (DAW/DAL = 0.621-0.679 mm for males, 0.76-0.89 mm for females) (Fig. A-33). Nevill (2007, 2009) measured the length and width of the dorsal apotome of members from *Culicoides* (*Avaritia*) Fox and found no significant difference between males and females. However, these measurements do not include the entire length of the DA. As stated in Borkent (2014), the ventral margin of the DA is hard to define. The area between the DA and head makes an hourglass shape (Fig. A-8C). This study uses the narrowest part of the hourglass as the definitive ventral margin of the DA. This dimorphism needs further study throughout *Culicoides* to determine its distribution.

As first found by Brad Mullens laboratory, the subbasal pores of the respiratory organ appear to be closed and covered by cuticle, and the pores at the apex appear to be cross shaped (Fig. A-13C and D). Abubekerov (2014) found that *C. sonorensis* prefer to pupate at or just below the water line at the margin of ponds or other moist habitats, and that the aforementioned morphological features may provide a superior means to sustain air contact in their environment. It would be interesting to conduct an SEM study to compare the respiratory organ of species with similar pupation behaviors and pupal habitats. SEM studies have shown that *Dasyhelea bilineata* Goetghebuer and *D. necrophila* Spinelli and Rodriguez have X-shaped apical and subapical pores (Dominiak 2012, Ronderos et al. 2003), *D. pseudolacustris* Díaz and Spinelli has somewhat elongated, sausage-shaped apical and subapical pores (Díaz et al. 2013), *D. eloyi* Díaz & Ronderos has a more square-shaped apical pores (subapical pores not shown) (Díaz et al. 2013), *Forcipomyia bromelicola* (Lutz) have squared to somewhat X-shaped apical pores (Marino et al. 2010), *Culicoides albomaculus* Root and Hoffman (Huerta et al.

2001) have oval-shaped openings at the respiratory organ apex and open subapical pores, *C. bambusicola* Lutz has apical slit-like pores (Ronderos & Spinelli 2000) and *Stilobezzia rabelloi* Lane has slit-like (more apical) to nearly circular subbasal pores (Borkent & Craig 2001). Although the information is highly limited, the presence of open subapical or subbasal pores in Forcipomyiinae, Dasyheleinae, at least one *Culicoides*, and a member of the Ceratopogoninae (*Stilobezzia*), suggests that the covered subbasal pores of *C. sonorensis* is a derived condition. Unfortunately, this character state can only be seen with an SEM. With further studies, we may be able to determine distribution of the sealed state and provide evidence of a monophyletic group within *Culicoides*. The shape of the pores varies and may have phylogenetic value. Further study is needed.

The chaetotaxy of abdominal segments of *C. sonorensis* (and generally in the subfamily Ceratopogoninae) follow a general pattern in which segment 1 (only the tergite is evident), segment 2 (only the tergite is evident), segment 3-7, and segment 8 show differences, with segments 1 and 8 showing reduced numbers of sensilla and segment 1 and 2 with reduced lateral setae size. As partially discussed by Borkent (2014), the accuracy of the specific naming of some sensilla is questionable. The overall similarity and placement of sensilla on these reduced segments is the basis of the specific numbering. For example, the two dorsal setae on segment 8 are named D-8-VIII and D-9-VIII because there are relatively similar to those on segment 7.

One major goal of this study was to complete the most extensive description of any Ceratopogonidae pupa. There is value in looking at the entire specimen rather than

only features previously deemed important. It was also advantageous to examine specimens using both SEM and compound light microscopy. Both methods have benefits, and together allowed most features to be photographed and labeled for *C. sonorensis*. Some campaniform sensilla were very difficult or impossible to see using SEM, though this study was able to definitively prove that the D-7-I campaniform sensillum is on the anterior margin of tergite 1 near D-3-I and D-2-I (Figs. A-14 and A-15), a synapomorphy of the genus (Borkent 2014). The fourth abdominal segment is typically the only abdominal segment described in species descriptions. However, the chaetotaxy for segments 1, 2, 4, and 8 show differences and there are further, possibly subtle, differences between each of segments 3-7 that cannot be determined until further comparative work is completed. Certainly, the relatively complete descriptions of pupae of Culicidae are illustrative of the value of this added information (e.g. Belkin et al. 1970). It would therefore appear to be useful to compare the chaetotaxy and other features of the whole pupa throughout the genus to identify distinguishing characteristic states and synapomorphies.

Historically, *Culicoides* have often been described from females, with secondary attention paid to the males. This clearly reflects human concerns regarding Ceratopogonidae as pests and vectors of disease agents. Subsequently, some collecting methods primarily (or only) sample females, further limiting taxonomic understanding. A more complete understanding of the morphology of species includes not only the two adult sexes, but also the immatures, an area requiring substantial development in Ceratopogonidae (Borkent 2014). Current *Culicoides* surveying techniques (Mayo et al.

2012, Schoenthal 2015) are usually focused on trapping the adult females. However, these methods collect very few, if any, males, and limit the total number of species collected from a given area. Studying and understanding the immature stages of *Culicoides* would improve our understanding of the features, distribution, habitats, and broader systematics of a given species or group including the possible discovery of new synapomorphies. It also provides the prospect of the discovery of new species.

The process for collecting and rearing most Ceratopogonidae pupae is relatively straightforward (Borkent 2014), requiring extraction by floatation from suitable substrates (e.g. mud, detritus on the margins of various aquatic habitats, wet mosses, dung, rotting vegetation) and rearing in suitable containers. As such, there is ample room for further study of the pupal stage in the life history of *Culicoides*.

CHAPTER III

CULICOIDES ASSOCIATED WITH TEXAS DEER BREEDING FACILITES

Introduction

The goal of this chapter was to determine the species of *Culicoides* associated with Texas deer breeding facilities and to start a genetic library of those species. This study was completed in collaboration with Dr. Cassie Schoenthal, who was also studying *Culicoides* on deer breeding facilities (Schoenthal 2015). She collected all of the specimens for this study as well as her own. The two facilities where traps were set out for this study are located in Burleson and Grimes County. The distribution records of four species (*C. crepuscularis, C. haematopotus, C. multipunctatus,* and *C. sonorensis*) are reported from five other counties (Bowie, Hays, Kerr, Live Oak, and Montague) (Schoenthal, unpublished data).

In this chapter, a synopsis of each species is given, including locality, seasonality, larval habitats, adult feeding habits, a short discussion, and the COI sequences obtained (if available). Many of the locality records for each species are new county records. Most of the species collected in this study had little to no genetic information available. Using the same primer as the Barcode of Life project (Hajibabaei et al. 2006), seven species now have new COI gene sequences in NCBI BLAST databases (Benson et al 2012). There are studies that show the utility of using COI gene for barcoding different species of *Culicoides* (e.g. Pages et al. 2009, Ander et al. 2013, Augot et al. 2013, Bellis et al. 2013, and Nielsen and Kristensen 2015). Ander et al.

(2013) does note, however, that this method has an approximate 5% identification failure rate for separating taxa. Difficulties arise when separating species that are too similar.

Materials and Methods

Adult *Culicoides* were collected using CDC light traps (Bioquip #2836BQ) suspended from the outer gates of deer enclosures. Each trap was secured to an inverted Igloo insulated container (#00001795, Katy, TX). The drink spout of the Igloo container was left open during the trapping period to allow CO_2 to escape at a controlled rate. Traps were set out in the evening, loaded with dry ice, and picked up the following morning (Schoenthal 2015). Two traps were used at two deer breeding facilities: one in Burleson County (30°23'55.49"N, 96°26'33.00"W), the other in Grimes County (30°29'11.21"N, 96° 9'45.30"W). The sampling period was February - August 2014. Specimens were collected dry from each trap, labeled with the date and location, and stored in a 15cm Petri dish at 0°C. Specimens were examined using a Leica S6D dissecting microscope. For each sample, *Culicoides* were separated into morphotypes. Morphotypes for each date were placed into individual Eppendorf tubes containing 95% ethanol, and then stored at -20ºC. A series of each morphotype was slide mounted in order to determine species. The mounting technique used is outlined in detail in Borkent and Spinelli (2007). Each specimen was separated into body regions: head, thorax, abdomen, and wings. The wings were put directly into 15% acetic acid. Each specimen was given a number to track each body region through the mounting process to ensure the species were not mixed. The rest of the specimen was cleared using 8% potassium

chloride (KOH) and heated using a microwave (Borkent and Spinelli (2007) used a water bath to heat the KOH). Glass vials of KOH containing specimens (one specimen per vial) were twice heated for 10 seconds. The specimens were allowed to clear and cool for 5-10 minutes. The body regions then rejoined their corresponding wings in the acetic acid. The whole specimen was then transferred to 2-propanol, then finally to 100% clove oil. A 7" plastic painter's palette was used for this process (Fig. A-36). Each well of the palette was filled with approximately 15mL of either 15% acetic acid (A), 2-propanol (P), or 100% clove oil (C). Three specimens could be processed per palette. A thin wooden applicator stick with a Minuten Pin inserted into one end was used to transfer specimens between wells. The end of the pin was bent into a hook shape (Fig. A-36B) (Dr. Pete Teel, personal communication). A small amount of Canada balsam mounting medium (Sigma-Aldrich; C1795) was added to a slide in four places. The wings, head, thorax, and abdomen were placed in separate drops of mounting medium and a cover slip was placed over each. Each slide was labelled with the collection date, location, and species name. Slides were heated in an oven at 50°C for 48 hours and allowed to air dry for 72 hours (Borkent and Spinelli 2007). The slide-mounted specimens were examined with a Nikon Alphashot-2 YS2 compound microscope.

An additional set of each morphotype was sequenced to obtain the COI gene. DNA extraction was carried out using a modification of the Gentra Puregene Kit (#D-5500A) (Gentra Systems, Inc., Minneapolis, MN). Each specimen was added to an Eppendorf tube containing 100µL of Cell Lysis Solution and 1µL of Proteinase K. The specimens were not crushed in order to retain their morphological features. They were

incubated in a water bath at 55ºC overnight. The specimens were then cooled in a refrigerator at 0ºC for 20-30 minutes, after which 35µL of 8.0M ammonium acetate was added to each tube. The samples spun at 2200 RPM for seven minutes in an Eppendorf 5424 Centrifuge. The supernatant from each tube was removed and added to a corresponding tube containing 100µL of cold isopropanol and placed in the freezer at -20ºC for ten minutes. During this cooling time, 100µL of 95% ethanol was added to the original tube containing the exoskeleton of the specimen. These specimens were then mounted using the protocol listed above, with the exception of the KOH clearing step, as the Proteinase K cleared the specimen. The tubes containing the supernatant were centrifuged again at 2200 RPM for 5 minutes. The isopropanol was poured out of each tube and 400µL of 100% ethanol was added. They were centrifuged for another five minutes at 2200 RPM. The ethanol was poured out and the tubes were inverted and left to dry for several hours. The samples were then re-suspended in 50µL of 1 x TE buffer and stored in the refrigerator at 0ºC.

PCR was performed using a modified protocol by Shokralla et al. (2010) and a Bio-Rad T100 thermal cycler. For each sample, 1.0µL of template DNA was added to a mixture of 18.54µL, 0.06µL Taq, 0.20µL forward primer, 0.20 reverse primer, and 5.0µL 5x buffer. The primers used in this study were Forward 5' GGT CAA ATC ATA AAG ATA TTG G 3' and reverse 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' (Folmer et al. 1994) diluted to a concentration of 10.0 pmol. The samples were capped, added to the thermal cycler, and run at 95ºC for three minutes, then 35 cycles of 95ºC for one minute, 45ºC for ninety seconds, and 72ºC for two minutes, with a final step of 72ºC

for five minutes. Samples were allowed to cool either in the machine or refrigerator at 0ºC.

Gel electrophoresis was preformed using Bio-Rad Sub-Cell GT Agarose Gel Electrophoresis System. A 2.0% agarose gel with ethidium bromide was added the electrophoresis machine, and 0.8 TBE buffer was added to the fill line. 5.0µL of DNA template was combined with 1.0µL of dye, and then added to wells of the gel. The machine was run at 85 volts for 1.5-2.0 hours. Ethidium bromide was added in order to visualize the bands in a Bio-Rad Molecular Imaging Gel Doc XR+ Imaging System. Only the samples with clean bands of approximately 600-700 base pairs in length were taken through the rest of the sequencing process.

PCR clean-up was completed using EXOSAP-IT protocol. An aliquot was made using the formula (0.04µL EXOI, 0.4µL SAP, 1.56µL water) multiplied by the total number of samples, multiplied by four. For each sample, 8.0µL of the aliquot was added (20µL remained from gel electrophoresis, bringing the total volume to 28µL). Using a thermal cycler, samples were incubated at 37 º C for 15 minutes and 80 º C for 15 minutes.

The concentration of DNA was quantified and recorded using a Qubit 3.0 fluorometer and a Qubit dsDNA HS assay kit. 2.0µL of DNA template was added to 198.0µL of dsDNA HS buffer and 1.0µL of dsDNA HS reagent for each sample. The samples were then left at room temperature for two minutes. Using the dsDNA high sensitivity setting, each sample was inserted into the fluorometer and the concentration

was recorded in ng/µL. This data was used during the subsequent step to determine how much DNA template to add to each BigDye reaction.

BigDye reactions were completed using BigDye Terminator v3.1 Cycle Sequencer Kit and protocol. Two aliquots were made during this step: one using the forward primer, the other using the reverse primer. The aliquots consisted of 2.0µL BigDye terminator, 6.0µL DNA sequencing buffer III (Teknova Cat No: D1301), and 0.32µL of the primer, multiplied by the total number samples. Using 100µL tubes, 8.32µL of the aliquot was added, labeled to correspond to a DNA sample. The amount of DNA needed was determined by using the chart on page 2-6 of the manual. Roughly 12.0ng of template DNA (quantified previously) was added to the corresponding tube. A varying amount of deionized water was added to each tube as to make the final volume of each sample 20.0µL. Each tube was capped. The samples were mixed well using a GeneMate vortex machine and spun for 30 seconds in a centrifuge. Samples were placed in the thermal cycler and run at a thermal ramp to 96ºC and held for 60 seconds, then 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes. Samples were allowed to cool either in the machine or refrigerator at 0ºC.

To each sample, 5.0µL of 125mM EDTA and 60.0µL of 100% ethanol was added. The samples were recapped and inverted four times. They were allowed to incubate at room temperature for 15 minutes. The tubes were placed into an Eppendorf 5430 centrifuge and spun at 2200 RPM for 30 minutes. The tubes were then uncapped, placed inverted into a GeneMate plate centrifuge and spun for ten seconds, after which 60.0µL of 70% ethanol was added to each tube and recapped. The samples were spun

again at 2200 RPM for another 30 minutes. Again, the tubes were uncapped and spun inverted for 10 seconds in the plate centrifuge. Samples were air dried in an Eppendorf Vacufuge Plus at 30ºC for 15 minutes using the D-AL setting. Once each tube was completely dry, 10.0µL of HiDi formide was added to each.

The samples were then loaded onto an Olympus Plastics 96-well PCR plate and added to an Applied Biosystems Hitachi 3500 Genetic Analyzer. The protocol run on this machine was standard sequencing using POP7 buffer. The chromatograms produced for each sequence were imported into the program Sequencher 4.8 for alignment. COI sequences of 658 base pairs in length were produced and added to GenBank, along with the corresponding species identification and locality data. The slide mounted specimens associated with the genetic data from this chapter were deposited into the Texas A&M University insect collection (voucher #720).

Results

A total 269 *Culicoides* representing twelve species were collected from the two deer breeding facilities. The species and abundances were as follows: *Culicoides arboricola* (0.4%), *C. butleri* (0.4%), *C. crepuscularis* (0.7%), *C. haematopotus* (6.7%), *C. hinmani* (0.4%), *C. multipunctatus* (21.2%), *C. neopulicaris* (34.9%), *C. paraensis* (0.4%), *C. sonorensis* (19.7%), *C. stellifer* (2.6%), *C. stonei* (10.0%), *and C. variipennis* (2.6%). Partial COI gene sequences were obtained for five species of *Culicoides* collected in this study (*C. crepuscularis, C. multipunctatus, C. neopulicaris, C.*

sonorensis, and *C. variipennis*). Detailed synopses for each species collected from the Brazos Valley are as follows:

Culicoides arboricola

Distribution and Seasonality: Eastern United States and Canada; Ontario, Minnesota to Connecticut, south to Texas and Florida (Borkent and Grogan 2009). It is abundant year-round in the southeastern part of the United States, and can be found during the warmer months in its northern distribution (Blanton and Wirth 1979).

Texas Distribution and Seasonality: This species was found in Burleson County in early summer (1.VI.2014) for the first time.

Larval Habitats: The larvae can be found in tree holes, moist cavities of trees and stumps, or in any kind of wet wood debris (Wirth and Bottimer 1956).

Feeding of adult females: The majority of the literature indicates that this species feeds on birds and it is often associated with poultry facilities. There are two accounts of females biting humans in Florida (Blanton and Wirth 1979) and rabbits and birds in Virginia (Humphreys and Turner 1973).

Discussion: *Culicoides arboricola* was identified using Wirth et al. (1985) and Blanton and Wirth (1979). It was readily distinguishable from other species collected in this study by wing pattern and female genitalia. There is a pale spot at each of the apices of wing veins Cu_1, M_1, M_2, and M_{3+4}. The wing pattern is similar to *C. butleri* although *C. arboricola* has both a rudimentary spermatheca and spermathecal ring.

Only one specimen was collected throughout the entire sampling period. This is a rare species in Louisiana (Blanton and Wirth 1979), and Texas could be the far western margin of its distribution. Blanton and Wirth (1979) and Wirth and Bottimer (1956) commonly collected this species in Florida using light traps.

Culicoides butleri

Distribution and Seasonality: Southwestern United States and northern Mexico; Arizona to Texas, south to Nuevo León. It can be found May to August (Borkent and Grogan 2009, Virgil et al. 2014).

Texas Distribution and Seasonality: This species was found in Burleson County during the summer (10.VII.2014) for the first time.

Larval Habitats: The larval habitat is unknown.

Feeding of adult females: Biting records are unknown for this species. Virgil et al. (2014) suggest that because this species has sensilla coeloconica on all thirteen flagellomeres, female *C. butleri* most likely feed on birds.

Discussion: *C. butleri* was identified using Wirth et al. (1985) and Wirth and Hubert (1960). As previously mentioned, the wing pattern of *C. butleri* is fairly similar to that of *C. arboricola*, however, *C. butleri* lacks both a rudimentary spermatheca and a spermathecal ring.

Only two specimens have ever been collected in Texas. In 1960, Robert Jones reared a long series of *Culicoides* from cacti in Kerrville, Texas (Wirth and Hubert 1960), but *C. butleri* was not one of the species discovered. Jones (1961) placed *C.*

butleri in the *copiosus* group, implying the larval habitat to be rotting cacti, though only adults in light traps have ever been collected. Atchley (1967) did an extensive survey of the *Culicoides* of New Mexico using light traps, and again, *C. butleri* was not among the species collected. Texas could be the northeastern border for this species, as this species is most likely geographically constricted to regions with cacti, if that is in fact the larval habitat.

Culicoides crepuscularis

Distribution and Seasonality: This species is one of the most common and widespread species of *Culicoides* in North America; ranging from southern Alaska to Nova Scotia, south to Costa Rica and Bermuda (Borkent and Grogan 2009, Blanton and Wirth 1979). This species is more abundant in warmer regions from April to October, with peak populations in the summer (Blanton and Wirth 1979).

Texas Distribution and Seasonality: *Culicoides crepuscularis* was collected in Bowie, Burleson, Grimes, Hays, Kerr, Live Oak, and Montague counties from May through October (22.V.2014 – 29.X.2014). This species was collected from most traps and locations fairly regularly, though not in large numbers (Schoenthal, unpublished data).

Larval Habitats: This species breeds in most freshwater soil habitats suitable for *Culicoides* (Jones 1961). *Culicoides crepuscularis* has been collected and reared from pond margins, stock tank overflows, sewage lagoons, septic tank effluents, roadside ditches, marshy meadows, stream margins, livestock watering troughs, polluted mud,

and cattle hoof prints (Blanton and Wirth 1979). Williams (1956, 1957) report this species in both freshwater and saltwater habitats in Bermuda.

Feeding of adult females: There are only two reports of this species feeding on humans (Blanton and Wirth 1979); however, females have been recorded biting a multitude of different bird species. It is often associated with poultry facilities.

Discussion: *Culicoides crepuscularis* was identified using Wirth et al. (1985) and Blanton and Wirth (1979). It was readily distinguishable from other species collected in this study by wing pattern and female genitalia. This was one of three species collected with only one spermatheca. The other two are *C. variipennis* and *C. sonorensis*. However, the latter two species have a curved, "C-shaped" spermatheca, while *C. crepuscularis* has an oval-shaped spermatheca.

Schoenthal (2015) reported the presence of Epizootic Hemorrhagic Disease (EHD) virus in this species. This is the first report of any Nearctic species other than *C. sonorensis* and *C. variipennis* as a potential vector of the virus. This will be addressed further in the discussion.

Culicoides haematopotus

Distribution and Seasonality: Widespread; British Columbia to Nova Scotia, U.S.A., Mexico, Honduras (Borkent and Grogan 2009). Some studies found this to be the most abundant species in light trapping collections (Wirth and Bottimer 1956, Snow et al. 1957). This species can be collected from spring until fall.

Texas Distribution and Seasonality: This species was collected in Bowie, Burleson, Grimes, Hays, Kerr, and Montague counties from March to July (18.III.2014 – 30.VII.2014).

Larval Habitats: Like *C. crepuscularis*, this species will breed in most freshwater soil habitats suitable to many species of *Culicoides* (Jones 1961). It has been collected and reared from pond and stream margins, mud, sand bars, and riverside pools (Blanton and Wirth 1979).

Feeding of adult females: This species is categorized as generalist feeder by Hair and Turner (1968), and not much is known about its host preferences. There are reports of females biting humans (Blanton and Wirth 1979). Wirth and Bottimer (1956) collected large numbers of this species at a poultry yards, and as suggested in Virgil et al. (2014), the presence of sensilla coeloconica on all thirteen flagellomeres suggests that the species is primarily ornithophilic.

Discussion: *Culicoides haematopotus* was identified using Wirth et al. (1985) and Blanton and Wirth (1979). It was readily distinguishable from other species collected in this study by wing pattern, and male and female genitalia. Females of this species have pale spots at the very apex of the wing, two spermathecae with a rudimentary spermatheca and spermathecal ring. Males have serrated paramere tips and bumps on the lateral arms of the aedeagus.

This species was the most abundant species collected in the Edwards Plateau Region from light traps (Wirth and Bottimer 1956). In the current study, *C. haematopotus* was collected regularly, though not in large numbers.

Culicoides hinmani

Distribution and Seasonality: United States; Wyoming and Utah to New York, south to Colorado, Texas, and Florida (Borkent and Grogan 2009). This species is active from May until October, though there were reports of collections in Florida as early as April and as late as December (Blanton and Wirth 1979).

Texas Distribution and Seasonality: This species was collected in Burleson County in July (16.VII.2014) for the first time.

Larval Habitats: This species has been reared from tree holes and moist tree cavities from many locations throughout its distribution (Blanton and Wirth 1979).

Feeding of adult females: Females of this species have been recorded feeding on small mammals, man, and in a few instances birds (Blanton and Wirth 1979).

Discussion: *Culicoides hinmani* was identified using Wirth et al. (1985) and Blanton and Wirth (1979). It was readily distinguishable from other species collected in this study by wing pattern, female genitalia, and eyes separation. The wing pattern of the female adult is closest to that of *C. crepuscularis*, though it is easily distinguishable due to its two spermathecae and contiguous eyes.

In this study, only one specimen was collected from the traps, however, the biology of this species would account for the low quantity of specimens. Snow et al. (1957) found this species to be primarily diurnal. The traps used in this study ran only during the night.

Culicoides multipunctatus

Distribution and Seasonality: Southern United States and Mexico; Kansas and Missouri, south to Texas and Alabama, Mexico (Morelos). It seems to be more common in the southern portion of its distribution (Wirth and Bottimer 1956).

Texas Distribution and Seasonality: This species was collected from Burleson, Grimes, Hays, and Live Oak counties from April until August (8.IV.2014 – 14.VIII.2014). Wirth and Bottimer (1956) found this species to be prevalent throughout the Edwards Plateau region, with the earliest record February 26th and the latest November 4th.

Larval Habitats: Adults of this species have been reared from mud collected at pond margins (Wirth and Bottimer 1956).

Feeding of adult females: There are no records of the feeding habits of this species.

Discussion: *Culicoides multipunctatus* was identified using Wirth et al. (1985), Malloch (1915), Atchley (1970), and Phillips (2015). It was the most distinctive of the species collected, as it was the only members of the subgenus *C.* (*Selfia* Khalaf) collected. Members of *C.* (*Selfia*) have no wing pattern, unsclerotized spermathecae, fused parameres, and a "trident-like" aedeagal tip. *Culicoides multipunctatus* is the only species in this subgenus found east of the Rocky Mountains.

Schoenthal (2015) detected the presence of EHD and Bluetongue virus in this species. This is further addressed in the discussion below. This species was the second most abundant species collected in Burleson County and the most abundant species in Grimes County. Though the feeding habits of the adult female are unknown, its

prevalence in CO_2 light traps could suggest a possible mammalian host. Further studies are needed in order to confirm this theory.

Culicoides neopulicaris

Distribution and Seasonality: Southern United States, Mexico, and Central America; Texas and Louisiana, south to Costa Rica (Borkent and Grogan 2009). Wirth and Bottimer 1956 collected this species in Kerrville, TX, but not in the much more arid area of Sonora, TX. Central Texas could be the northwestern border for this species. *Culicoides neopulicaris* was collected in light traps from spring to fall (Wirth and Bottimer 1956).

Texas Distribution and Seasonality: This species was collected from Burleson and Grimes counties from May until July (07.V.2014 – 30.VII.2014). This was the most abundant species from the traps in Burleson County, with peak numbers in June and July. This is new county record for both Burleson and Grimes counties.

Larval Habitats: Larval habitats are unknown.

Feeding of adult females: There are no records of the feeding habits of this species.

Discussion: *Culicoides neopulicaris* was identified using Wirth et al. (1985) and Wirth (1955). Adults are readily distinguishable from those of all other *Culicoides* collected in this study by wing pattern alone. Most of the wing is pale with a few dark distinct marks near the wing margins.

This species was collected in large numbers using CO_2 light traps, which suggests a mammalian host. Other members of this subgenus are reported as mammalophilic (Vigil et al. 2014).

Culicoides paraensis

Distribution and Seasonality: United States, Mexico and Central and South America; Colorado, Nebraska, Pennsylvania and Wisconsin, south to Louisiana and Florida, south to Argentina (Borkent and Grogan 2009). Little is known of this species' seasonality in the US. However in Argentina, it can be collected during the spring and fall, with peak populations in the fall (Veggiani-Aybar et al. 2011).

Texas Distribution and Seasonality: One specimen was collected in Grimes County on 30.VII.2014. Both Wirth and Bottimer (1956) and Vigil et al. (2014) conducted extensive surveys in areas of Texas but did not report this species. This is the first record of this species in Texas.

Larval Habitats: This species is mainly found in tree holes and moist wood debris, with reports of it being reared from sap and rotten vegetation (Blanton and Wirth 1979).

Feeding of adult females: Snow et al. (1957) reported this species feeding during the day, then intensifying at dusk. There are many reports of females relentlessly biting humans (Blanton and Wirth 1979). Humphreys and Turner (1973) reports females feeding on rabbits, turkeys, and chickens.

Discussion: *Culicoides paraensis* was identified using Wirth et al. (1985) and Blanton and Wirth (1979). It was readily distinguishable from other species collected in this

study by the wing pattern, female genitalia, and antennal segments. This species is similar to *C. stellifer* in wing pattern and with the absence of sensilla coeloconica on flagellomeres 9-13. However, *C. paraensis* lacks a spermathecal ring, has a deep pit located on the third palpal segment, and additional pale spots on the wings (Blanton and Wirth 1979).

In Argentina, *C. paraensis* is a known to vector of the Bluetongue virus as well as Filariasis (Veggiani-Aybar et al. 2011). Its capacity to transmit disease pathogens should be taken into account in further epidemiological studies.

Culicoides sonorensis

Distribution and Seasonality: British Columbia, Alberta, Montana, and South Dakota, south to California, Kansas and Texas; scattered localities east of the Mississippi River in the Midwest, southeast, and middle Atlantic states: Illinois, Ohio, Virginia, Maryland, Kentucky, Tennessee, North Carolina, Louisiana, Alabama, Florida, Mexico (Borkent and Grogan 2009, Holbrook et al. 2000, Vigil et al. 2014). With such a wide range, seasonality can vary. In the northern US and southern Canada, this species is seen from May to August (Lysyk 2007, Lysyk and Danyk 2007). In southern California, this species can be collected year round (Gerry et al. 2001).

Texas Distribution and Seasonality: This species was collected in Bowie, Burleson, Grimes, Hays, Kerr, Live Oak, and Montague counties. *C. sonorensis* was regularly collected from traps from February through October (19.II.2014 – 29.X.2014), with strong peaks in August (Schoenthal, unpublished data). The species seasonality of *C.*

sonorensis (as *C. variipennis*) reported in Wirth and Bottimer (1957) is nearly identical to the findings of this study.

Larval Habitats: This species is heavily associated with feed lots and stock yards and can be found in stock tank overflows, rainfall collections, wetlands, pasture sloughs, lakes, waste water, and mud and sand at pond margins. Very high numbers are reported in polluted mud, sewage effluents, and moist areas receiving large amount of organic materials (Blanton and Wirth 1979, Schmidtmann et al. 2011, and Wirth and Bottimer 1956). Schoenthal (2015) shows strong evidence that *C. sonorensis* larvae will flourish in moist habitats with introduced feed runoff.

Feeding of adult females: Females of this species feed on large ruminants such as cattle, sheep, deer, and horses (Howarth et al. 2008). There is a lone report of this species feeding on chickens (Blanton and Wirth 1979).

Discussion: *Culicoides sonorensis* was identified using Wirth et al. (1985), Wirth and Jones (1957), and Holbrook et al. (2000). This species is distinguishable from all other *Culicoides* collected in the study with the exception of *C. variipennis*, by wing pattern and female genitalia. Both *C. sonorensis* and *C. variipennis* have a “C-shaped” single spermatheca. Females of these species are more easily distinguishable from each other. The third palpal segment of *C. sonorensis* is greatly swollen, but not so in *C. variipennis*. The males of these two species are harder to discern. The aedeagus membrane of *C. sonorensis* is densely covered by spicules. The aedeagus membrane of *C. variipennis* can be bare to somewhat covered in spicules. *Culicoides sonorensis* was one of the most abundant species collected in this study.

Culicoides stellifer

Distribution and Seasonality: United States and Canada; Ontario to Nova Scotia, all of the continental US other than Washington and Oregon (Borkent and Grogan 2009). This species can be collected from spring until fall with two population peaks in May and August (Blanton and Wirth 1979).

Texas Distribution and Seasonality: This species was collected from Grimes County, once in April, and twice in late July. Wirth and Bottimer (1956) collected this species in Kerr County from April to early October. This is a new county record for Grimes County.

Larval Habitats: *C. stellifer* larvae can be found in moist soil, wet leaf litter, mud from pond margins, lake margins, and overflow from streams (Blanton and Wirth 1979 and Wirth and Bottimer 1956).

Feeding of adult females: Humphreys and Turner (1973) collected one female specimen from a turkey, and three from a goat. Smith et al. (1996) collected this species in large numbers from a captive deer.

Discussion: *Culicoides stellifer* was identified using Wirth et al. (1985) and Blanton and Wirth (1979). This species is readily distinguishable from other species collected in this study by wing pattern, and third palpal segment. This species' wing pattern is similar to that of *C. paraensis*, though with fewer pale spots, and *C. paraensis* lacks the pit on the third palpal segment of *C. stellifer*.

Culicoides stellifer was not collected regularly in this study. However, the dates in which it was collected correlates to their peak populations (Blanton and Wirth 1979). It is likely that this species was collected due to a population spike in the area.

Culicoides stonei

Distribution and Seasonality: Utah to South Dakota, south to New Mexico; Texas and Oklahoma (Borkent and Grogan 2009, Wirth and Blanton 1971). This species is common in arid grasslands of the southwest (Wirth and Blanton 1971) and along the gulf coast (Jones 1961). This species had been collected from May until August, though one recorded specimen was collected as late November.

Texas Distribution and Seasonality: This species was collected in Burleson County with relatively high numbers during the summer months (5.VI.2014 – 30.VII.2014). This is a new county record for Burleson County.

Larval Habitats: Jones (1959) lists both salt and alkaline soil habitats in Texas, and found slightly denser populations in grassy pools near the gulf coast. This species has been reared from stream margins in Utah (Wirth and Blanton 1971).

Feeding of adult females: In the slide mounted material examined by Jones (1961), two of the female specimens were labeled as “biting deer”, and “from White-tailed deer.”

Discussion: *Culicoides stonei* was identified using the Wirth et al. (1985) and Wirth and Jones (1961). It is distinguishable from the other species collected in this study by wing pattern and female genitalia. The only other species collected in this study with no wing

pattern was *C. multipunctatus*, though it has an unsclerotized spermatheca, whereas *C. stonei* has two spermathecae in addition to the rudimentary one.

The relative abundance of this species collected from CO_2 light traps, seems to corroborate the records of this species feeding on deer.

Culicoides variipennis

Distribution and Seasonality: United States and Canada; British Columbia, Washington to Nova Scotia, south to Montana, Texas, Louisiana and Florida, Mexico (Blanton and Wirth 1979 and Borkent and Grogan 2009). This species was collected in the spring and summer months in Florida (Blanton and Wirth 1979).

Texas Distribution and Seasonality: This species was sparsely collected in Burleson and Grimes counties in early spring and summer (18.III.2014, 12.VI.2014 – 16.VII.2014).

Larval Habitats: The larvae of *C. variipennis* have been collected and reared from mud, polluted water, manure, pond margins, stock tank seep, and livestock runoff (Blanton and Wirth 1979, Schmidtmann et al. 1998)

Feeding of adult females: This species is strongly associated with livestock, and the females will feed on large ruminants with at least one record of it them biting humans (Blanton and Wirth 1979, Jamnback 1965).

Discussion: *Culicoides variipennis* was identified using the Wirth et al. (1985) and Blanton and Wirth (1979). This species is readily distinguishable from other species collected, with the exception of *C. sonorensis*, by wing pattern and female genitalia.

Culicoides variipennis has a distinct wing pattern and one C-shaped spermatheca. This species lacks the swollen third palpal segment found in *C. sonorensis*.

Texas has long been known as a habitat of *C. variipennis* and *C. sonorensis* (Wirth and Jones 1957 and Holbrook et al. 2000), though the abundance of these two species is unknown. This study suggests that *C. sonorensis* is the more abundant species in the Brazos Valley. Roughly seven times as many *C. sonorensis* were collected than *C. variipennis*, though as the breeding sites are known, further pupal surveys should be conducted to confirm this.

Discussion

The main goal of this chapter was to document the species of *Culicoides* associated with Texas deer breeding facilities, specifically targeting those species in which the females take blood meals from large mammals. This study and Schoenthal (2015) are the first surveys of *Culicoides* species in southeast Texas. However, it is highly unlikely that this is a complete survey of the total number of species for this area. As seen in *C. hinmani*, a species that feeds during the day was extremely rare using this collecting technique. Other diurnal species could have been excluded all together. Species of *Culicoides* that rely heavily on visual cues or other host signals would also not have been collected using these traps. Mosquitos can use a wide range of biotic factors during host selection such body heat, body mass, other olfactory cues, and visual conformation of the host or specific body regions (Takken and Verhulst 2013). In host selection, CO_2 is often the first (long-range attraction) cue, followed by a positive short-

range response to a host (Bidlingmayer 1994, Takken and Verhulst 2013). Those species most likely to feed on deer were caught in relatively large numbers (>6.0% of the total specimens collected), with the exception of *C. variipennis*. Most of the bird-feeding species were rarely collected with this method (<1% of the total specimens collected). This suggests that these ornithophilic species are using other means to locate hosts, and could possibly be repelled by an abundance of CO_2.

The livestock identified as vertebrate hosts of biting flies have only recently been introduced to the US (in an evolutionary sense). Species of *Culicoides* have either changed hosts or feed on the introduced vertebrates in addition to their native host. More biting records for a number of *Culicoides* species collected in this study are needed in order to determine their current host preferences.

Another problem encountered in this study was the overwhelming majority of female specimens collected. Males of various species of *Culicoides* often have distinct genitalia, and can aid in identification. In order to collect more males, traps should be set closer to larval habitats, in additions to rearing pupae (Borkent, personal communication). Any future surveying attempts would benefit greatly with the addition of a variety of collecting techniques (light-only traps, pupal rearing, and live traps). This would certainly increase the total number of *Culicoides* collected, as well as the number of species.

This study was unable to obtain sequences from every species collected. In some instances, only one specimen was available for a species. Some of the samples could have also degraded and dried out making it impossible to obtain a sequence. This study

also attempted to sequence partial 16S sequences, but was not successful, possibly due to corrupt primers.

EHD virus was found in *C. crepuscularis* and Bluetongue and EHD viruses were found in *C. multipunctatus* (Schoenthal 2015). The *Culicoides* species tested in Schoenthal (2015) were pooled into samples of a maximum of five specimens per species, per collection date. In testing for viral components, all specimens were destroyed. This method does not allow for verification of the results post-testing. However, this is the first repot of these viruses in both of these species. This does not indicate a capacity to transmit these disease pathogens, but it does offer insight into the feeding habits of these species.

There are no biting records for *C. multipunctatus*, though in this study, large numbers of this species where collected using CO_2 light traps, suggesting this species could be feeding on large ruminants. However, *C. crepuscularis* females are known to be primarily ornithophilic, and it is not clear how a ruminant virus was contracted by bird a feeding species. There are two possibilities that would explain this result and could lead to interesting future work. The simplest is that this species may feed on both birds and mammals, even if the latter is on rare occasions. There are two records of *C. crepuscularis* females feeding on man (Blanton and Wirth 1979). Another possibility is an undiscovered, avian reservoir host for EHD. There has long been the question of how this virus survives the winter, and the mechanisms allowing this are poorly understood (Sperlova and Zendulkova 2011). A reservoir host with a longer viremia than the ruminant hosts would offer a possible explanation to this conundrum (Wilson et al.

2008). It is important to note that there are other possibilities for how the virus overwinters, such as trans ovarian transmission in the vector, trans placental infection in the host, small populations of *Culicoides* surviving the winter with the virus, or mechanical tick vectors (Bouwknegt et al. 2010, Sperlova and Zendulkova 2011, and Wilson et al. 2008), but none have been demonstrated. In any case, the possibility of *C. crepuscularis* species are feeding on both birds and mammals should be investigated further.

CHAPTER IV

A PHYLOGENETIC ANALYSIS OF THE SUBGENUS *CULICOIDES* (*MONOCULICOIDES*)

Introduction

The distribution of *Culicoides* is nearly worldwide (it is absent from New Zealand), with 1355 described species, 31 subgenera, 38 species groups, and 176 unplaced species. In recent years phylogenetic hypotheses using molecular data have been proposed for very limited numbers of *Culicoides* (generally including less than 10 species), limited to a specific region (restricted areas within Europe). For a comprehensive list of many of these studies, see Table 3 in Harrup et al. (2014). These molecular methods have nearly all incorporated only a single gene (generally CO1). As such, there have been no morphologically based cladistical analyses of any species of *Culicoides*. Determining the evolutionary relationships between individuals provides a basis for making more logical comparisons of the included species regarding their biology (adult and larval habitats, feeding behavior, life cycle, vector competency).

Culicoides as a genus forms an unresolved trichotomy, with two small genera, *Washingtonhelea* Wirth and Grogan (one species from California) and *Paradasyhelea* Macfie (11 species from Australia, France, Argentina, and Washington) and together these form the tribe Culicoidini. Further outgroup relationships are well established (Borkent 2014), with Culicoidini forming the sister group of remaining Ceratopogoninae, and this subfamily being the sister group of Forcipomyiinae +

Dasyheleinae. All these taxa are the sister group of the Leptoconopinae, which includes only two genera, *Leptoconops* Skuse and *Austroconops* (Borkent 2014)

The subgenus *C.* (*Monoculicoides*) includes 23 species which are primarily Palearctic, with one Oriental species, *C. longlinensis* Yu, and one Southern African species, *C. cornutus*. There are currently seven species of *C.* (*Monoculicoides*) recognized in the Nearctic Region: *C. sonorensis*, *C. variipennis*, *C. occidentalis*, *C. riethi*, *C. grandensis*, *C. shemanchuki* and, newly recognized from North America, *C. stigma* (Grogan and Lysyk 2015). That paper synonymized *C. gigas* Root and Hoffman, a species previously recognized as Nearctic, with *C. riethi*, a species previously restricted to the Palearctic Region.

The main goals of this chapter were to examine evidence that *Culicoides* (*Monoculicoides*) is monophyletic and to determine the relationships of the included species. Previous studies have pointed out that members of this subgenus have unusual and/or distinctive features: large larval pharyngeal complex (Kettle and Lawson 1952), singular spermatheca and fused parameres (Khalaf 1954), and bifid aedeagus (Fox 1955). These, and other features, were incorporated into the study below.

Khalaf (1954), when first proposing the subgenus *C.* (*Monoculicoides*), considered the taxon to include a much broader group, including four species groups, namely: the *nubeculosus* group, *fulvithorax* group, *guttifer* group, and *crepuscularis* group. Today we know these groups to include species from the subgenera *C.* (*Monoculicoides*), *C.* (*Beltranmyia* Vargas), *C.* (*Meijerehelea* Wirth and Hubert), and *C.* (*Trithecoides* Wirth and Hubert). Members of *C.* (*Monoculicoides*) are restricted to those

that Khalaf (1954) called the *nubeculosus* group, with the exception of *C. hegneri* Causey, which currently placed in *C.* (*Meijerehelea*).

Several recent molecular studies have included members of the subgenus *C.* (*Monoculicoides*), and whereas single genes can be inaccurate when used for phylogenetic analysis, the consistent grouping in each paper point to the possible monophyly of the subgenus (Perrin et al. 2006, Ander et al. 2013, and Sarvasova et al. 2014). Perrin et al. (2006), studying the ITS_1 gene of French species placed *C. punticollis*, *C. nubeculosus*, *C. parroti*, and *C. variipennis* (an included Nearctic species) into a single clade. Sarvasova et al. (2014) used COI gene sequences to analyze *Culicoides* species from Slovakia with results identical to those in Perrin et al. (2006). Ander et al. (2013) used the COI gene to perform a rigorous analysis across ten subgenera. Though not all species of certain subgenera were grouped together, those of *C.* (*Monoculicoides*), *C. nubeculosus*, *C. riethi*, and *C. stigma* were placed in a single node. The data generated by these studies provide evidence of the genetic similarities within the subgenus *C.* (*Monoculicoides*).

Understanding the evolutionary history of this subgenus is of particular importance because it contains both primary vectors of the disease-causing agents of Bluetongue and Epizootic Hemorrhagic Disease in North America. The subgenus *C. (Monoculicoides*) was selected for this study in part because it includes species with significant ecological and economic importance as disease pathogen vectors, but additionally because the subgenus had prior evidence of monophyly. This study includes

trees from both a genetic based analysis and morphological phylogenetic analysis of the subgenus.

Methods and Materials

A morphological character matrix was created using Microsoft Excel 2010. Three exemplar species of a select number of subgenera and all 23 species of *Culicoides* (*Monoculicoides*) were included in the matrix. The subgenera, *C*. (*Beltranmyia*) and *C*. (*Meijerehelea*) were selected due to the grouping in Khalaf (1954); all other subgenera were randomly chosen as a small representation of the genus. The phylogeny of the basal lineages of Ceratopogonidae is well-established by Borkent (1995, 2000, and 2014), which allowed this study to select appropriate outgroups. Based on the most recent phylogeny in Borkent (2014), the genus *Austroconops* Wirth and Lee was used as an outgroup as it represents the earliest extant lineage in the family, and is considered to have a predominance of plesiomorphic features.

After an extensive literature review and thorough examination of many specimens of *C*. (*Monoculicoides*), a list of 15 characters was scored for 47 taxa. Each taxon was scored for each character, “0” denotes the absence of a character state, “1” denotes the presence of a character state, and “?” denotes missing information about a character state. The character matrix is shown in Table B-1. The excel file was saved as a tab delineated text file, edited, and converted into a Nexus file using the text editor Notepad++. The Nexus file was imported into the program Tree Analysis Using New Technologies (TNT) version 1.1 – Willi Hennig Society Edition. An unweighted,

traditional analysis was run using tree bisection reconnection (TBR) while collapsing trees after each search. This was replicated three times using an increase of 500 sequences per search. The tree buffer was filtered for suboptimal trees, with a minimum branch length of zero, and then condensed. Another two traditional searches were performed, again using an increase of 500 sequences per search. The tree buffer was filtered and condensed. A final traditional search was done using all trees from the RAM. The tree buffer was filtered and condensed a final time. A strict consensus tree was created from the trees in the buffer. The consensus tree was analyzed using the program Mesquite version 3.04 (Maddison and Madison 2015) and visualized using the program Fig Tree version 1.4.0. Phylogenetic Analysis Using Parsimony (PAUP* 4.0b10) was also used for parsimony analysis of the data set in Table B-1 (Swofford 2003). This program was used to calculate the consistency index and retention index of the consensus tree as well as the individual characters. It was also used to visualize the nodes at which an unambiguous state change occurs (Fig. A-39).

The molecular analysis was based on specimens sequenced in Chapter III as well as sequences obtain from two species of *Forcipomyia* Lenz (*F. squamipes* (Coquillett), and *Forcipomyia* sp.), one species of *Dasyhelea* Kieffer (*Dasyhelea* sp.), and five other *Culicoides* (*C. impunctatus* Goetghebuer*, C. nubeculosus, C. occidentalis, C. riethi, and C. shemanchuki*), resulting in a total of 38 sequences (Table B-4). Each specimen was designated with a number to denote locality data. These numbers serve only to differentiate sequences of the same species. The sequences from Table B-4 were imported into the program MEGA version 6.06: In MEGA, a jModelTest (Posada 2008)

selection analysis was run to determine the optimal model for phylogenetic analysis. A maximum likelihood statistical analysis and a general time reversible model with gamma distribution rate and invariant sites was used with a bootstrap analysis (value = 2000) to construct a cladogram. The constructed tree was visualized using the program Fig Tree version 1.4.0 (Figs. A-37 and A-38). Members of the genus *Forcipomyia* and *Dasyhelea* were also collected and sequenced in Chapter III, and were used as outgroups for this study.

Character States

The character matrix (Table B-1) is based on characters considered to be of phylogenetic significance as they vary between subgenera of *Culicoides* and between various members of *C.* (*Monoculicoides*), as listed below. The numbers before each character state correlates to those in Table B-1. The consistency index (C.I.) and retention index (R.I.) are listed for each character state.

1. Pupa without dark pigmentation on the pedicel of the respiratory organ (0); pupa with this dark pigmentation (1) (Figs. A-3A, A-7B, and A-13B) (C.I. = 1.0, R.I. = 1.0).

Borkent (2014) published an extensive study of the pupae of Ceratopogonidae in which this character trait was absent except for the members of *C.* (*Monoculicoides*) that were examined. This character state is unique to at least Ceratopogonidae. Though there was a lack of slide mounted pupae and limited pupal descriptions, all *C.*

(*Monoculicoides*) pupae examined showed this apparently derived feature (*C. grandensis, C. nubeculosus, C. occidentalis, C. riethi, C. shemanchuki, C. sonorensis, and C. variipennis*).

2. Adult females, eyes touching or nearly touching (0); female eyes broadly separated (1) (C.I. = 0.5, R.I. = 0.875).

Three character states are present within *Culicoides*, eyes touching, eyes nearly touching, and eyes broadly separated. However, eye separation is variable and can be difficult to interpret, and this characters state oscillates within Ceratopogonidae. Most of the higher members of this family have eyes widely separated, though sometimes abutting (Wirth and Grogan 1988). The earliest lineages of Ceratopogonidae have varying character states. *Austroconops* species have eyes that are narrowly separated, whereas members in Forcipomyiinae and Dasyheleinae [sister taxon to Culicoidini (*Culicoides* and *Paradasyhelea*)] have eyes abutting. In *Culicoides*, broadly separated female eyes are a trait shared by all *C.* (*Monoculicoides*) as well as all but one *C.* (*Beltranmyia*). *Culicoides crepuscularis* Malloch has eyes that are narrowly separated.

3. Adult males, bumps not present on the lateral arms of the aedeagus (0); bumps present (1) (C.I. = 1.0, R.I. = 1.0).

The plesiomorphic state is present in all species used in this study except for those in the subgenus *C.* (*Diphaomyia* Vargas) and *C. rarus* Dasgupta. The derived condition is most likely unique within *Culicoides*.

4. Adults males, medial notch not present on the apex of the 9th abdominal segment (0); medial notch present (1) (C.I. = 0.2, R.I. = 0.0).

A medial notch is defined as an indention at the apex of the 9th abdominal segment (as in Fig. A-30B). The apomorphic character state is present in multiple subgenera such as *Culicoides* (*C.* (*Amossovia* Glukhova), *C.* (*Beltranmyia*), *C.* (*Culicoides*), *C.* (*Hoffmania* Fox), and *C.* (*Silvaticulicoides* Glukhova), including all members of *C.* (*Monoculicoides*) except *C. longlinensis*.

Some species of *Culicoides* have the plesiomorphic state, but much like eye separation, this character can be difficult to interpret within Ceratopogonidae. It is complicated by the diverse modifications of the 9th tergite throughout the family, as present in *Ceratopogon* Meigen, an early lineage within the Ceratopogoninae. One solution to the ambiguousness of this character could be to better define the medial notch, possibly in its association with the lobe accessories of the 9th abdominal segment.

5. Adult males, aedeagus not bifurcated (0); aedeagus bifurcated (1) (C.I. = 0.5, R.I. = 0.9).

The plesiomorphic state is present in the early lineages of Ceratopogonidae (Leptoconopinae and Forcipomyiinae) as well as all subgenera of *Culicoides* other than *C.* (*Monoculicoides*). All but one member of this subgenus, *C. grandensis*, has a bifurcated aedeagus. The apomorphic state is also seen in some *Dasyhelea* and all

Ceratopogon. This indicates the character is homoplastic within the family, having evolved independently in *C.* (*Monoculicoides*) and in some other genera.

6. Adult males, parameres not fused or only fused at the base (0); parameres entirely fused medially (1) (C.I. = 0.25, R.I. = 0.7).

The parameres of all the species of the subgenus *C.* (*Monoculicoides*) are fused, however, *C. longlinensis* and *C. heiheensis* Li, Zhang, and Liu are alone in having parameres fused only at the base, possibly placing these two species as sister taxa to the other members. The rest to the species in *C.* (*Monoculicoides*) have medially fused parameres. Two other species of *Culicoides*, *C.* (*Meijerehelea*) *hegneri* and *C.* (*Beltranmyia*) *knowltoni* Beck, also have the apomorphic state. Basal fusion is present in *Austroconops* and some *Dasyhelea*. Two separate (unfused) parameres are seen in the majority of the subgenera of *Culicoides*.

7. Adult females, spermatheca not curved (0); spermatheca curved (1) (C.I. = 1.0, R.I. = 1.0).

The apomorphic state is present in *C. sonorensis, C. occidentalis, C. variipennis, C. longicollis,* and *C. nubeculosus* (Fig. A-39). This trait is considered derived, as it is unique within Ceratopogonidae.

8. Adult females, spermatheca without protrusion (0); spermatheca with protrusion (1) (C.I. = 1.0, R.I. = 1.0).

This character state is most certainly derived as is it not seen in any other member of Ceratopogonidae. This protrusions can range from a large, fat mass attached to the spermatheca (character state 9) seen in *C. combinotheca* and *C. parroti*, to a finger-like extension which is present in *C. digitalis, C. helveticus, C. stigma,* and *C. xinghaiensis*.

9. Adult females, spermatheca without large protrusion (0); spermatheca with large protrusion (1) (C.I. = 1.0, R.I. = 1.0).

The apomorphic character state is only seen in *C. combinotheca* and *C. parroti*. This character is derived as it is not seen in any other Ceratopogonidae.

10. Adult females, with two spermathecae (0), with one spermatheca (1) (C.I. = 0.25, R.I. = 0.5).

There are three subgenera of *Culicoides* in which the apomorphic state is present in [*C.* (*Monoculicoides*), *C.* (*Beltranmyia*), and *C.* (*Meijerehelea*)]. One spermatheca is a character state present in many other Ceratopogonidae genera.

11. Adult females, spermathecal duct with narrow opening (0); spermathecal duct with wide opening (1) (C.I. = 0.33, R.I. = 0.8).

The apomorphic character state is only seen in two subgenera of *Culicoides* [*C.* (*Monoculicoides*) and *C.* (*Meijerehelea*)], and seems to be derived as it is not present in

any other species of *Culicoides*. Two members of the subgenus *C.* (*Monoculicoides*), *C. helveticus* and *C. xinghaiensis*, have the plesiomorphic state.

12. Adults females, without dark spot on wing just posterior to arculus and CuA vein of wing (0); with dark spot (1) (C.I. = 1.0, R.I. = 1.0).

The apomorphic state is likely a synapomorphy for *C.* (*Monoculicoides*), though this study was unable to study some Chinese species. Despite this, 16 of the 23 species of this subgenus do have the apomorphic character state. Three members of this subgenus, *C. stigma*, *C. parroti*, and *C. longlinensis* have a sharp reduction in this pigmentation. This seems to be derived as it is not seen in any other Ceratopogonidae, and could be an excellent synapomorphic character for *C.* (*Monoculicoides*) once all members of the subgenus has been examined.

13. Adults females, with second radial cell of wing not fully pigmented (0), second radial cell fully pigmented (1) (C.I. = 0.25, R.I. = 0.0).

This is another variable character state throughout all of Ceratopogonidae. Fully pigmented second radial cells are seen in *Forcipomyia*, *Dasyhelea*, *Brachypogon* Kieffer, and is not seen in *Austroconops* species. In the higher lineages of Ceratopogonidae, however, the second radial cell is elongated, making the comparison more difficult. Within *Culicoides*, the apomorphic state is present in the subgenera *C.* (*Amossovia*), *C.* (*Beltranmyia*), *C.* (*Diphaomyia*), *C.* (*Meijerehelea*), and *C. (Monoculicoides*).

14. Pupal dorsal thoracic seta D-5-T much shorter than D-1-T and D-2-T (Fig. A-10C) (0); pupal dorsal thoracic setae D-1-T, D-2-T, D-5-T all stout and nearly equal in length (1) (C.I. = 1.0, R.I. = 1.0).

The character state represents an autapomorphy found only in *C. shemanchuki*. This character is most certainly derived as it is not shared with any other members of Ceratopogonidae.

15. Adult females, with sensilla coeloconica only on flagellomeres 1-8 (0); sensilla coeloconica on flagellomeres 9-13 (1) (C.I. = 0.33, R.I. = 0.71).

Sensilla coeloconica are present on flagellomeres 1-8 in all members of the subgenus *C.* (*Monoculicoides*). All other *Culicoides* examined in this study except two [*C.* (*Diphaomyia*) *baueri* and *C. rarus*], have sensilla coeloconica present on flagellomers 9-13.

Results

The 15 character states described above and distributed in the matrix in Table B-1 produced 56 equally parsimonious trees. The differences between the trees primarily regarded the placement of the unresolved members of the subgenus *C.* (*Monoculicoides*). A strict consensus tree is shown in Figs. A-38 and A-39 (length = 34, C.I. = 0.4412 and

R.I. = 0.7654). Character states changes are indicated on appropriate branches in Fig. A-38, and unambiguous state changes are denoted by nodes 33 – 40 in Fig. A-39.

Seven distinct clades were identified (shown with Roman numerals in Fig. A-38) with unambiguous states changes shown in nodes 33 – 40 (Fig. A-39), rooted with an *Austroconops* outgroup. Clade I, node 39, contains all the subgenera of *Culicoides* used in this study, except *C.* (*Diphaomyia*) and *C.* (*Monoculicoides*). Clade II, node 33, contains both *C.* (*Diphaomyia*) and *C. rarus*. Clade III, node 38, contains all of the members of the subgenus *C.* (*Monoculicoides*), with Clades IV – VII, nodes 34, 35, 36, and 37, showing further resolutions within the subgenus.

Not included in the monophyletic clade containing *C.* (*Monoculicoides*) is the species *C. rarus*. This indicates that it not should not be included in the subgenus as is not only lacks all of the synapomorphic characters states, but many of the shared character states as well. Reassignment to the subgenus *C.* (*Diphaomyia*) is suggested as a possibility due to the shared synapomorphy of bumps on the lateral arms of the aedeagus. This species is further discussed later.

This phylogeny differs from the results of a recent study pertaining to *C.* (*Monoculicoides*). Grogan and Lysyk (2015) provide a grouping of all Nearctic species of *C.* (*Monoculicoides*) other than the *variipennis* complex into the newly formed *nubeculosus-stigma* complex, based on ovoid-shaped spermatheca, and reduced mandibular teeth and wing patterning. The present study found *C. nubeculosus* to be most closely related to the *variipennis* complex and *C. longlinensis* (node 34), and places *C. stigma* in a clade with mainly Palearctic species of *C.* (*Monoculicoides*) (node 37)

(Figs. A-38 and A-39). This indicates the possibility of the *nubeculosus-stigma* complex of Grogan and Lysyk (2015) as an invalid grouping.

Although the COI gene tree (Fig. A-37) does not refute the conclusion that the subgenus *Monoculicoides* is monophyletic, it does not provide strong support for it. The bootstrap value for this clade is under 50, and therefore not shown in Fig. A-37. This analysis was unable to differentiate sequences of *C. sonorensis* and *C. variipennis*.

Bootstrap analysis of the COI data lends support to *C. occidentalis* as less genetically similar to the other two species of the *variipennis* complex. This contradicts the results of Holbrook et al. (2000), who reported *C. variipennis* as the genetically less similar to *C. occidentalis* and *C. sonorensis*. Tabachnick (1992) conducted a similar analysis using the same species, but included *C. riethi* (as *C. gigas*) as an outgroup. The genetic similarity reported by Tabachnick matches what was found in this study.

Discussion

Khalaf (1954) grouped certain *Culicoides* species into one clade based upon having a singular spermatheca and paramere fusion, which he designates as the subgenus *Culicoides* (*Monoculicoides*). Khalaf further grouped the "nubeculosus group" (which included *C. nubeculosus, C. parroti*, *C. puncticollis, C. riethi, C. stigma,* and *C. variipennis*) and *C. hegneri*. The distinction was made that parameres of *C. hegneri* least resembled other members of this clade (in reference to basal fusion rather than medial fusion), and this species was later moved to *C.* (*Meijerehelea*).

Through the analysis of the character matrix phylogeny, this study found synapomorphies (pigmentation of the pupal respiratory horn base, bifurcated aedeagus, medial fusion of the parameres, a single spermatheca with a wide duct opening, and a dark spot on the wing posterior to the CuA vein) for the subgenus *C.* (*Monoculicoides*) In addition, several derived characters (curved spermatheca, small spermathecal protrusions, and large spermathecal protrusions) were discovered that offer further resolution of this subgenus, as well as an autapomorphy in *C. shemanchuki* (pupal dorsal thoracic setae D-1-T, D-2-T, D-5-T all stout and nearly equal in length). The phylogenetic relevance of several characters (eyes broadly separated, medial notch present, second radial cell fully pigmented, and the presence of sensilla coeloconica on flagellomeres 1-8) could not be determined, however, they remain character states seen in all members of the subgenus *C.* (*Monoculicoides*).

Clade II

In Fig. A-39 clade II is represented by node 33. This clade contains the subgenus *C.* (*Diphaomyia*) and *C. rarus*. Character 3 (1), bump on the lateral arms of the aedeagus, is most certainly a derived character state as it is not seen any other *Culicoides*. Members of this clade also lack a medial notch, character 4 (0), though this character is less reliable as it varies in the genus, and the present study was unable to determine the phylogenetic significance of this feature.

Clade III

In Fig. A-39 clade II is represented by node 38. This clade contains all of the members of the subgenus *C.* (*Monoculicoides*). Characters 2 (1), eyes broadly separated; 4 (1), medial notch present; 13 (1), second radial cell fully pigmented; and 15 (0), the presence of sensilla coeloconica on flagellomeres 1-8, are seen in all members of this subgenus. However, this study found these characters to be strongly variable within the genus Culicoides and consider them presently unreliable for phylogenetic analysis.

Five synapomorphies were identified within this clade. Even with some missing information characters 1 (1), pigmentation of the pupal respiratory horn base, and 12 (1), a dark spot on the wing posterior to the CuA vein, were found to be synapomorphic, as these characters were present in all *C.* (*Monoculicoides*) examined. Character 5 (1), bifurcated aedeagus, is present in all but one species of *C.* (*Monoculicoides*). In *C. grandensis*, there is a reversion to the plesiomorphic state of character 5. In addition, a trifurcated aedeagus is present in members of the subgenus *C.* (*Selfia*), a group not used in the present study. This character could be derived as it is not seen in any other *Culicoides* species, though further study is needed to determine if this is a true truncation of the aedeagus, or fusion of the lateral prongs. Within Ceratopogonidae, this study identified are three character states associated with the male parameres; basal fusion, medial fusion, and separation of the parameres. Character state 6 (1), medial fusion, is a derived condition present in all members of *C.* (*Monoculicoides*), except two *C. heiheensis* and *C. longlinensis*, and is unique within the family. The combination of a single spermatheca, character 10 (1), with a wide duct opening, character state 11 (1), is

present in all member of *C.* (*Meijerehelea*) and *C.* (*Monoculicoides*). There is evidence of homoplasy for these two characters within Ceratopogonidae, though they are not seen in any other *Culicoides* species.

Clade IV

In Fig. A-39 clade IV is represented by node 34. This clade contains *C. longicollis, C. nubeculosus, C. occidentalis, C. sonorensis,* and *C. variipennis*. Members of this clade all share character state 7 (1), spermatheca curved, which is derived within *C.* (*Monoculicoides*), and is not seen in any other subgenus of *Culicoides*. There may be even further resolution in looking at the shape of spermatheca, though this is not shown in the present study. *Culicoides nubeculosus* and *C. longicollis* are both Palarctic, and have rather oval-shaped spermatheca, whereas in the other members of this clade are Nearctic with an elongated, C-shaped spermatheca. This clade is strongly supported by the C.I. and R.I. values for character 7. The derived condition is also found in in the genus *Schizonyxhelea* Clastrier, which likely instances of homoplasy.

Clade V

In Fig. A-39 clade V is represented by node 37. This clade contains *C. digitalis, C. combinotheca, C. parroti, C. stigma, C. helveticus,* and *C. xinghaiensis*. Members of this clade all share character 8 (1), spermatheca with protrusion. In all but two species, discussed later, this protrusion is a finger-like extension off the apex of the spermatheca.

This character is derived and is not seen anywhere else in Ceratopogonidae. This clade is strongly supported by the C.I. and R.I. values for character 8.

Clade VI

In Fig. A-39 clade VI is represented by node 35. This clade contains *C. combinotheca* and *C. parroti*. These two species differ from other members of clade V based on character 9 (1), spermathecal protrusion large. In these species, the protrusion is a bulky (roughly ¼ the size of the spermatheca). This character seems to be a modification of character 8. This clade is strongly supported by the C.I. and R.I. values for character 9.

Clade VII

In Fig. A-39 clade VII is represented by node 36. This clade contains *C. helveticus* and *C. xinghaiensis*. These are the only two species of *C.* (*Monoculicoides*) with a narrow spermathecal duct opening, character 11 (0), a reversion to the plesiomorphic state. Within Ceratopogonidae, there is evidence of homoplasy in this character state.

Two more characters need to be discussed, though they are not included in the phylogenetic analyses. The first is the size of the pharyngeal complex in larvae. There have been few in-depth larval descriptions for Ceratopogonidae and only about 13% of *Culicoides* species are known as larvae. Five of the twenty-three *C.* (*Monoculicoides*) species in this study have a larval description (Borkent 2014). These species (*C. nubeculosus, C. riethi, C. stigma, C. sonorensis*, and *C. variipennis*) all have a large

pharyngeal complex (Kettle and Lawson 1952). This character state is not found in any other *Culicoides* (Glukova 1989 and Kettle and Lawson 1952). Further research should be conducted to confirm this probable synapomorphy

Additionally, the tips of the parameres seem to have some phylogenetic relationship within *Culicoides*. Paramere tips can be either smooth or serrated in appearance. Within most subgenera, this character state is consistent among members of the group. However, there are some exceptions to this, as there is variation within the same subgenus, as in *C.* (*Silvaticulicoides*). This inconsistency resulted in exclusion of the character from the matrix.

Though a phylogenetic relationship could not be established, several character states changes seen in Fig. A-38 are shared between *C.* (*Monoculicoides*), *C.* (*Beltranmyia*), and *C.* (*Meijerehelea*). Members of all three of these subgenera have only one spermatheca. Two members of the subgenus *C.* (*Monoculicoides*) have basal fusion of the parameres, a character state otherwise only seen in *C.* (*Beltranmyia*) and *C.* (*Meijerehelea*). A future study investigating these taxa is highly recommended.

This study shows strong evidence that *C. rarus* does not belong in *C.* (*Monoculicoides*). Dasgupta (1963) initially described this species and placed it in the subgenus *C.* (*Meijerehelea*). Gangopadhyay and Dasgupta (1998) later reported this species as a member of the subgenus *C.* (*Oecacta*) Poey. Finally, Yu et al. (2005) dissolved *C.* (*Meijerehelea*) and dispersed its members throughout the genus, placing *C. rarus* in *C.* (*Monoculicoides*). The constant reassignments suggest a thorough analysis of *C. rarus* and its placement in the genus has yet to be completed. Though the scope of

this study does not include the reassignment of *C. rarus*, it is clear that the species is not a member of *C.* (*Monoculicoides*) (Figs. A-38 and A-39). A close examination of *C.* (*Diphaomyia*) is suggested.

This study found the COI gene to be generally accurate for determining species, with the exception of *C. sonorensis* and *C. variipennis*, which were too genetically similar to be separated using this gene. This study does not report any phylogenetic analysis based solely on the COI gene tree (Fig. A-37). There are many examples where the use of a single gene to construct a phylogeny can yield inaccurate results (Beckenbach and Borkent 2003, Ander et al. 2013). Augot et al. (2013) performed a phylogenetic analysis of *C.* (*Monoculicoides*) using ITS_1, COI, and Cytb sequences. The tree created using COI sequences disagreed with the trees created using ITS and Cytb sequences. Further still, the latter two trees group *C. nubeculosus* with *C. puncticollis*, and did not group *C. stigma* and *C. parroti*. According the current study, this grouping is not the most parsimonious, and would suggest several derived conditions within the genus having evolved independently multiple times.

CHAPTER V

SUMMARY AND CONCLUSIONS

The main goal of this thesis project was to raise awareness of the gaps in knowledge surrounding *Culicoides* in Texas, more specifically, in the Brazos Valley. This study endeavored to increase knowledge of this group, specifically through examination of taxonomy, morphology, systematics, and genetics. Additionally, steps were made in order to understand the evolutionary history of the subgenus *C.* (*Monoculicoides*).

This study completed the first complete pupal description of *C. sonorensis*. This species was chosen because of its acute ecological and economic impact as the principle vector of BTV and its ability to vector EHDV in North America. An in-depth study was conducted using multiple forms of microscopy to complete a thorough description of the pupae. This included the first complete Scanning Electron Microscope images of the pupa of this species. These images allowed for morphological comparison with other Ceratopogonidae. Unique in its depth and thoroughness, this work has far-reaching implications in such areas as ecology, systematics, and vector biology and control. Results from this research concluded that the pupae of *C. sonorensis* are distinguishable from those of all other species of *Culicoides.* This is of particular interest because adult members of the *variipennis* complex are not so easily distinguished. This work provided new images, descriptions, and character traits that allowed these taxa to be separated

from one another. This could provide possible implications for disease management as these disease vectors can now be identified in the pupal stage.

The next objective was to complete a survey of *Culicoides* species found at Texas deer breeding facilities, give a species synopsis for each, and started a genetic library of North American species. The species synopses certainly do not reflect all species in Texas, but are a collection of pertinent information concerning *Culicoides* present at breeding facilities. This can be used in the future for identification and control of the pests themselves and of potentially control the diseases they transmit. DNA was extracted and sequenced in-house and resulting sequences, some new, were uploaded into NCBI BLAST databases. Additionally, this study improved upon existing data already present in GenBank. For some species, existing sequences were extended by up to 200 base pairs. This work has expanded the available information, though more projects such as this need to be undertaken and improved upon before we have a complete survey of *Culicoides* in Texas.

The final objective was to construct the evolutionary history of the subgenus *C.* (*Monoculicoides*). A phylogenetic tree was constructed through a morphological character matrix, and a neighbor joining tree was created using the genetic information previously obtained. Analysis of character based tree gave strong evidence that *C.* (*Monoculicoides*) is monophyletic. Genetic similarities also support the relation between species of *C.* (*Monoculicoides*). Additionally, it was found that the phylogeny does not support the placement of *C. rarus* in the subgenus. Though reassignment was outside the

scope of this project, evidence suggests a possible placement for the species in *C.* (*Diphaomyia*).

The lack of quality information and analysis concerning *Culicoides* is troubling. Huge gaps in the knowledge base are common, with fundamental pieces of information missing entirely. This is of particular concern, since this group causes hundreds of millions of dollars in damages and lost revenue each year in the United States alone. Through this study, an attempt was made to remedy some of this deficit of information. Special attention should be paid to the *Culicoides* of Texas, as new species could still exist within the state as large portions remain un-sampled. By closing these critical gaps in knowledge, a more complete understanding of *Culicoides* can be achieved.

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APPENDIX A

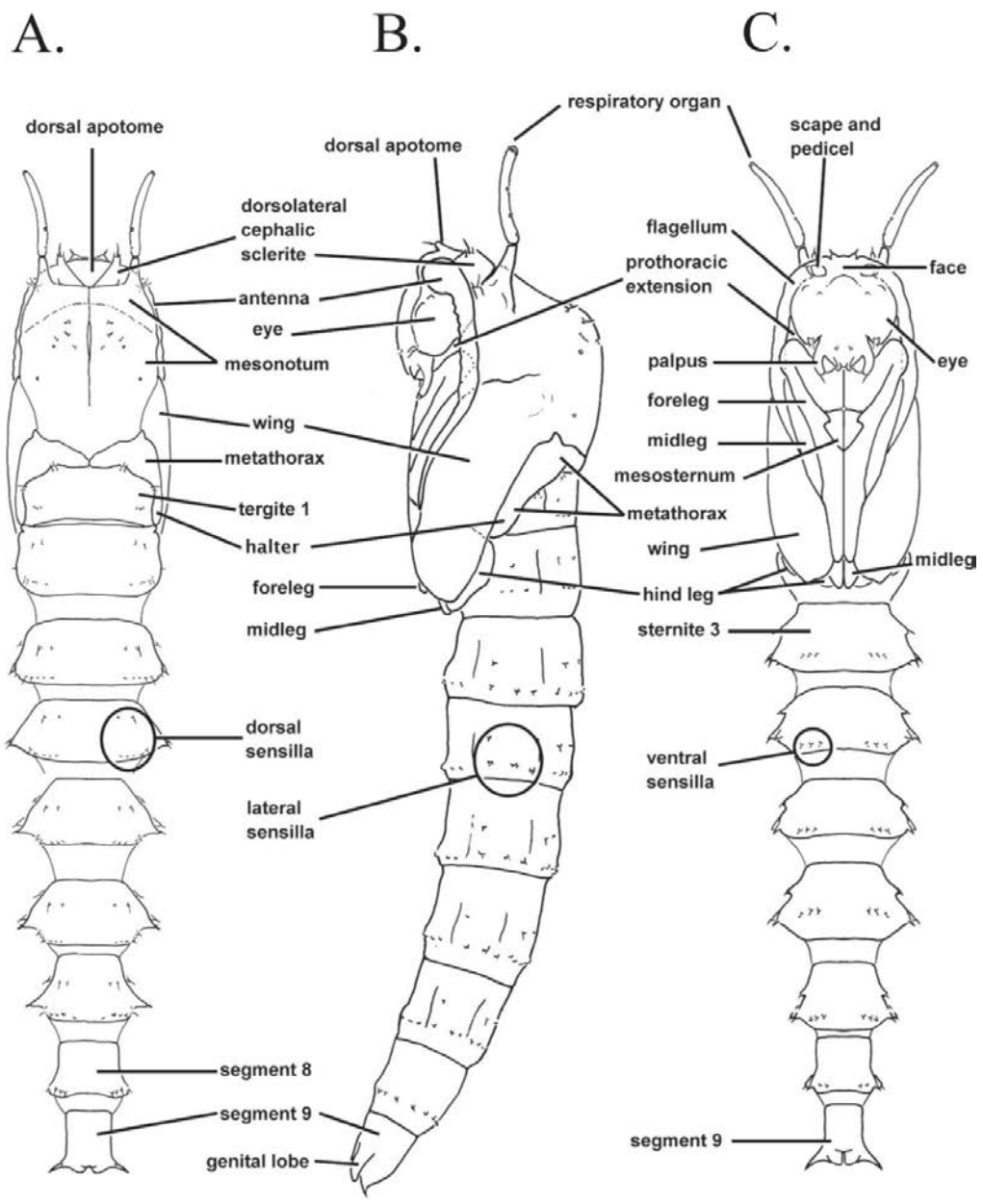

Fig. A-1. Habitus of pupae of *Culicoides sonorensis* (from Borkent 2012). (A) Female, in dorsal view. (B) Male, in lateral view. (C) Female, in ventral view. A and C have the abdominal segments separated by expanded membrane, not shown in B. Shagreen not shown.

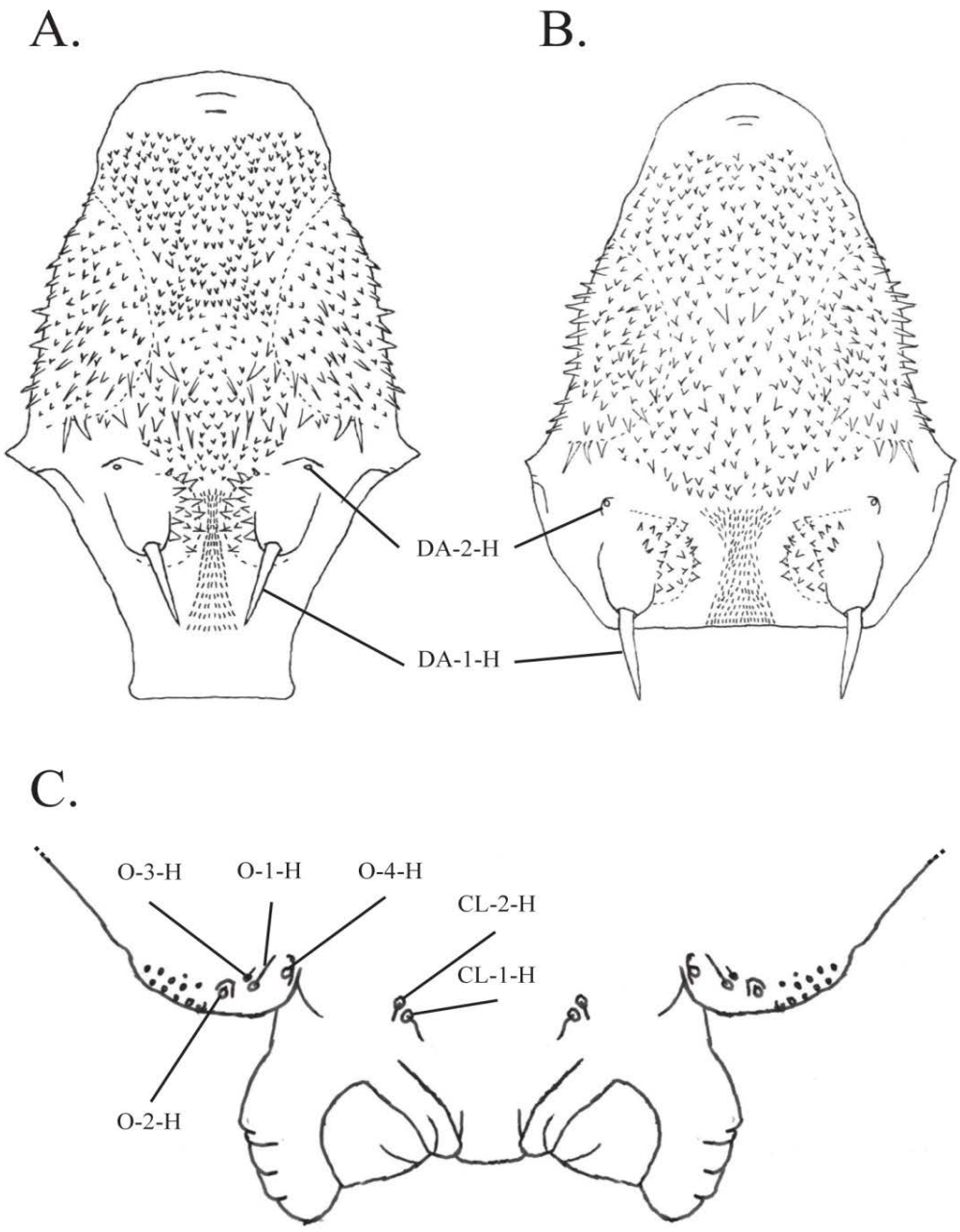

Fig. A-2. Structures of the pupal head of *Culicoides sonorensis.* (A) Male dorsal apotome, in anterior view. (B) Female dorsal apotome, in anterior view. (C) Mouthparts, in ventral view.

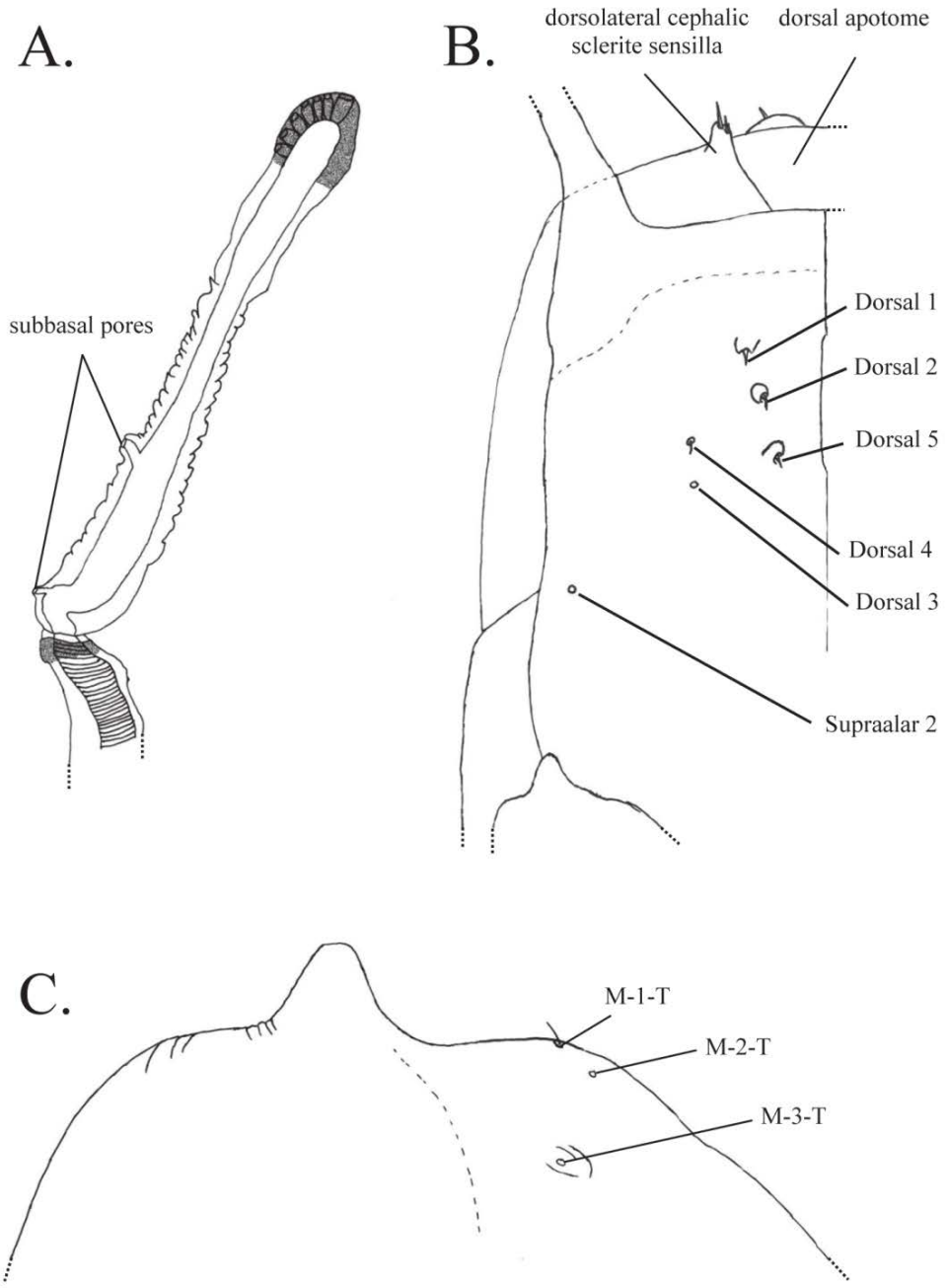

Fig. A-3. Structures of the pupal thorax of *Culicoides sonorensis*. (A) Respiratory organ, in dorsal view. (B) Dorsal setae, right side, in dorsal view. (C) Metathorax, left side, in dorsal view.

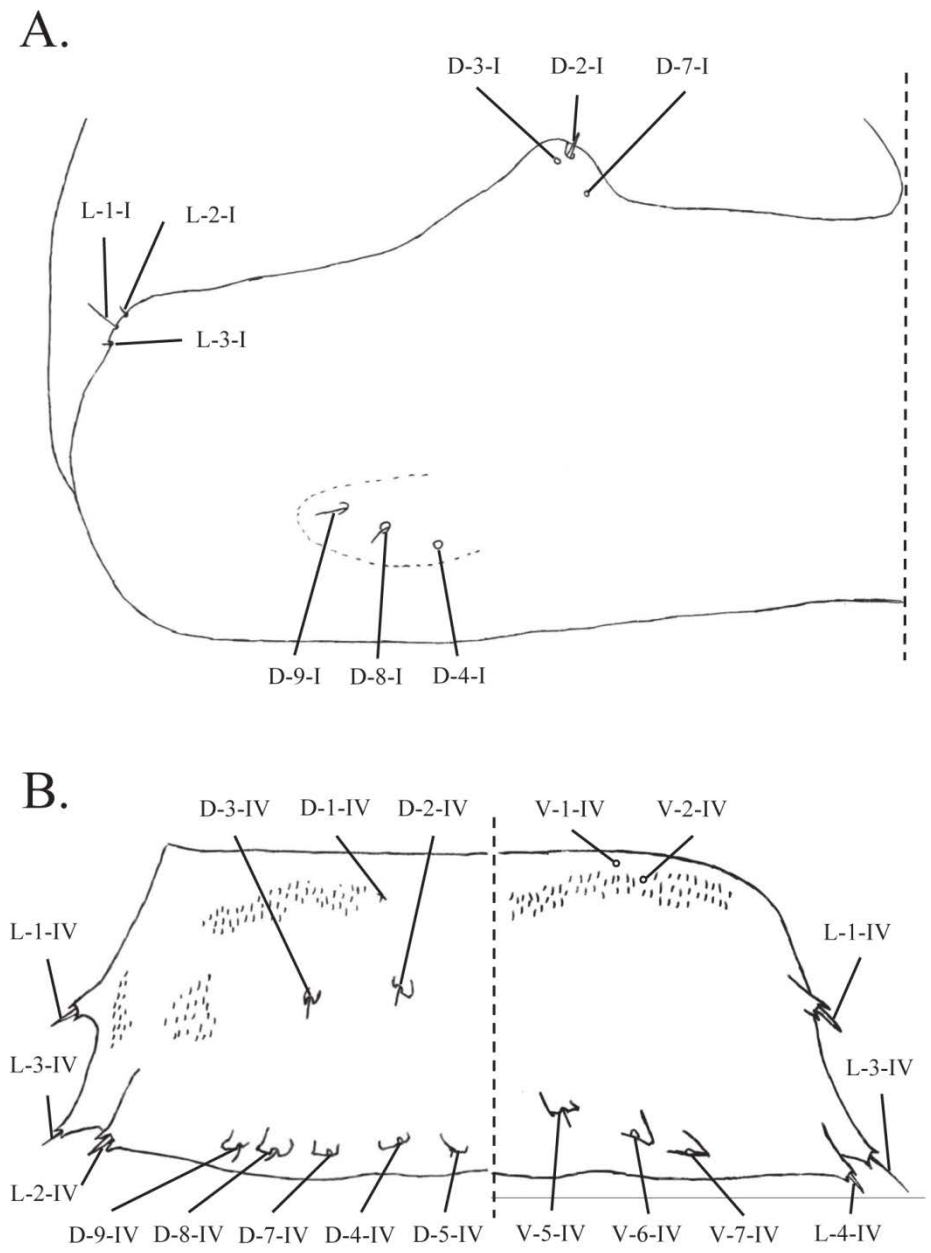

Fig. A-4. Structures of the pupal abdomen of *Culicoides sonorensis*. (A) Tergite 1, left side, in dorsal view. (B) Segment 4, tergite, left, in dorsal view; sternite, right, in ventral view.

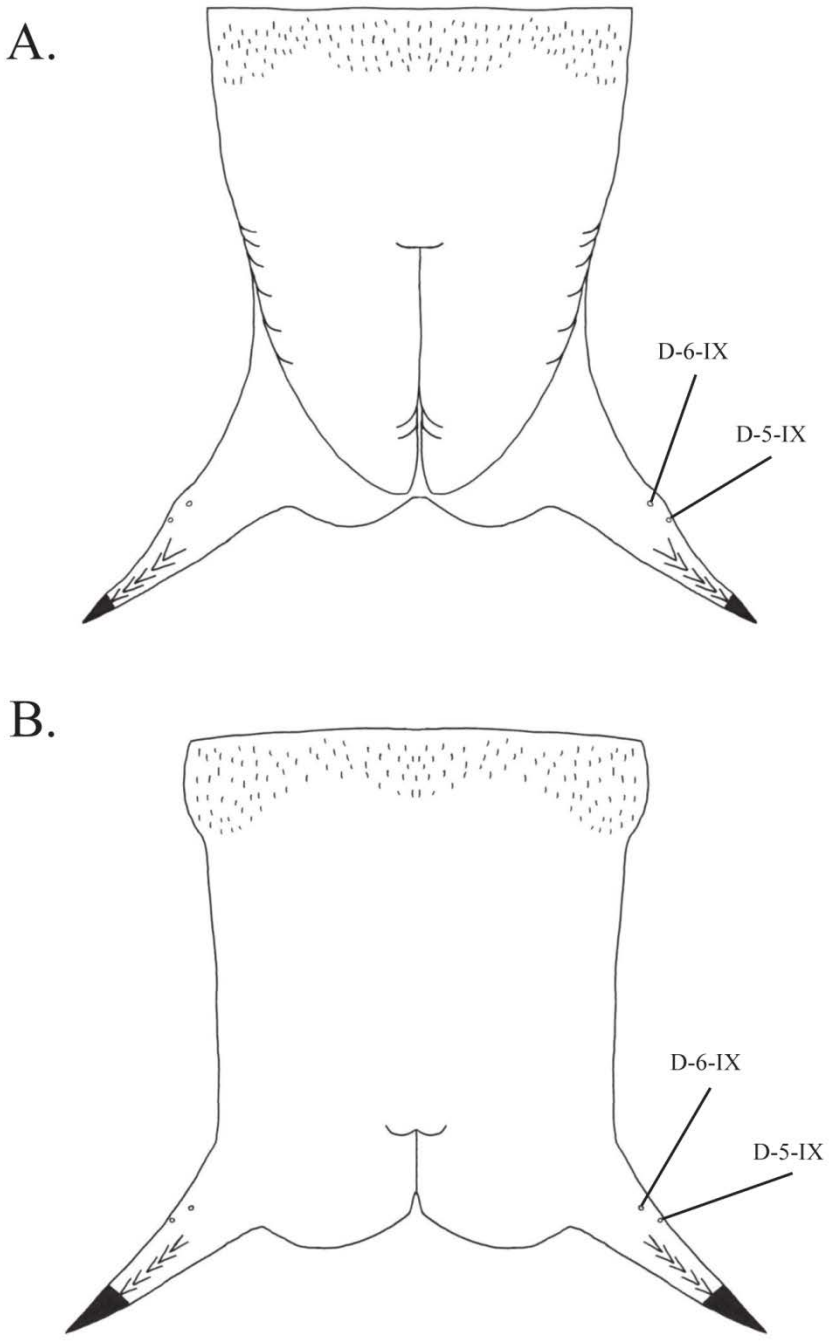

Fig. A-5. Segment 9 of *Culicoides sonorensis* in ventral view. (A) Male. (B) Female.

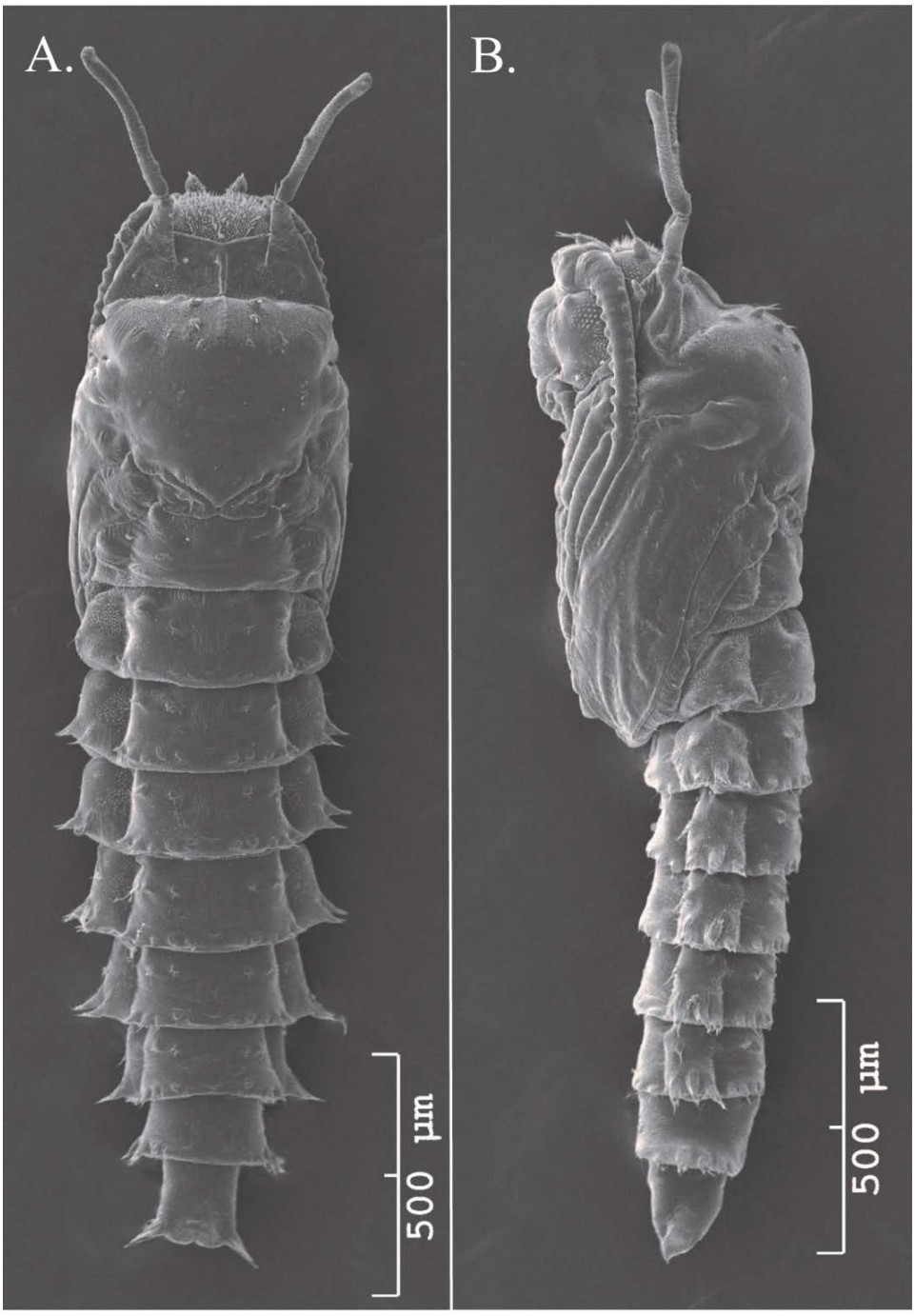

Fig. A-6. Habitus of the pupa of *Culicoides sonorensis* with SEM. (A) Female, in dorsal view. (B) Male, in left lateral view.

Fig. A-7. Habitus of the pupa of *Culicoides sonorensis*, in ventral view. (A) Female, with SEM. (B) Male, with compound microscope.

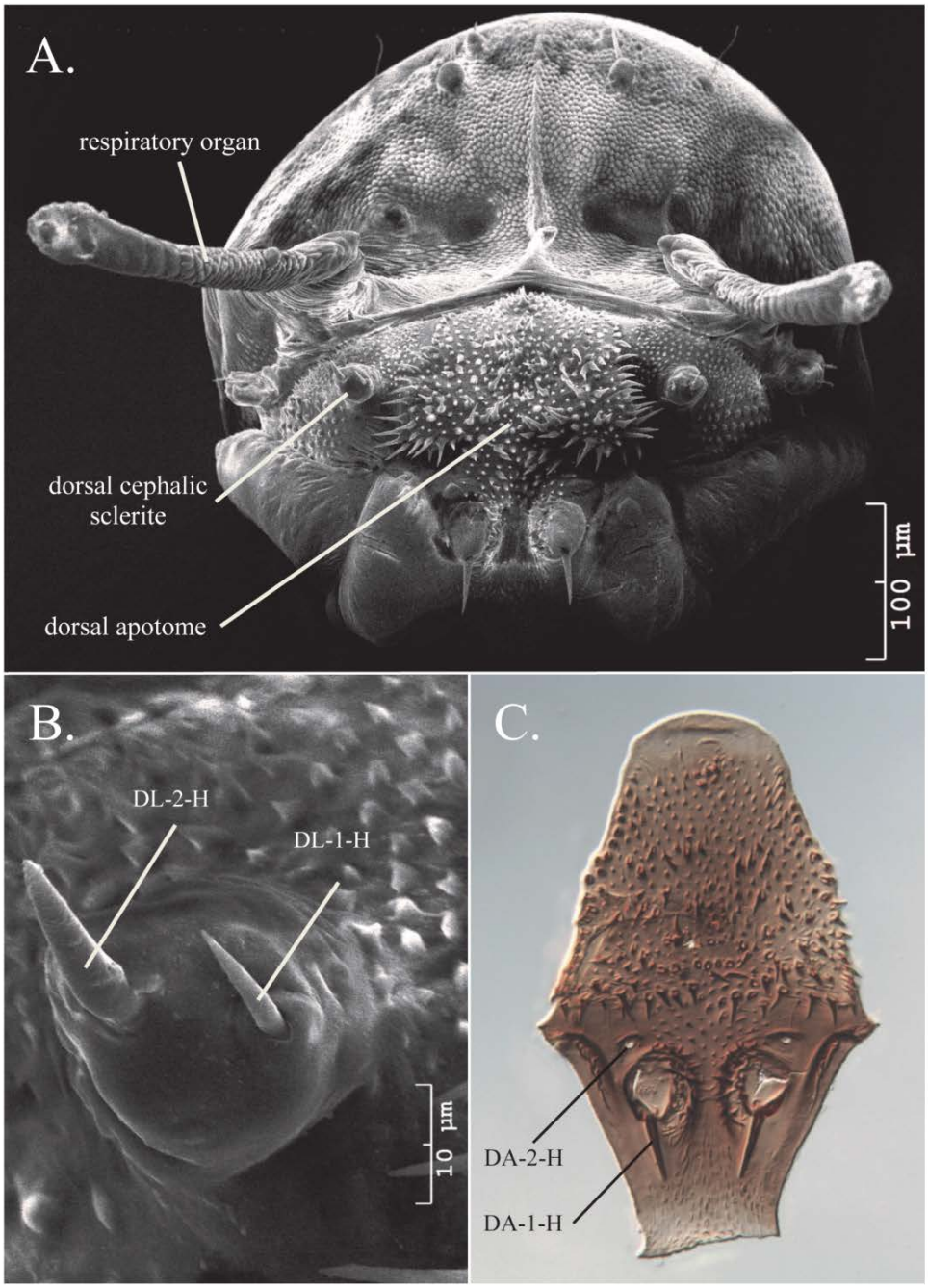

Fig. A-8. Structures of the pupal head of *Culicoides sonorensis*. (A) Head and thorax, in anterior view with SEM. (B) Dorsolateral cephalic sclerite, in anterior view with SEM. (C) Male, dorsal apotome, in anterior view with compound light microscope.

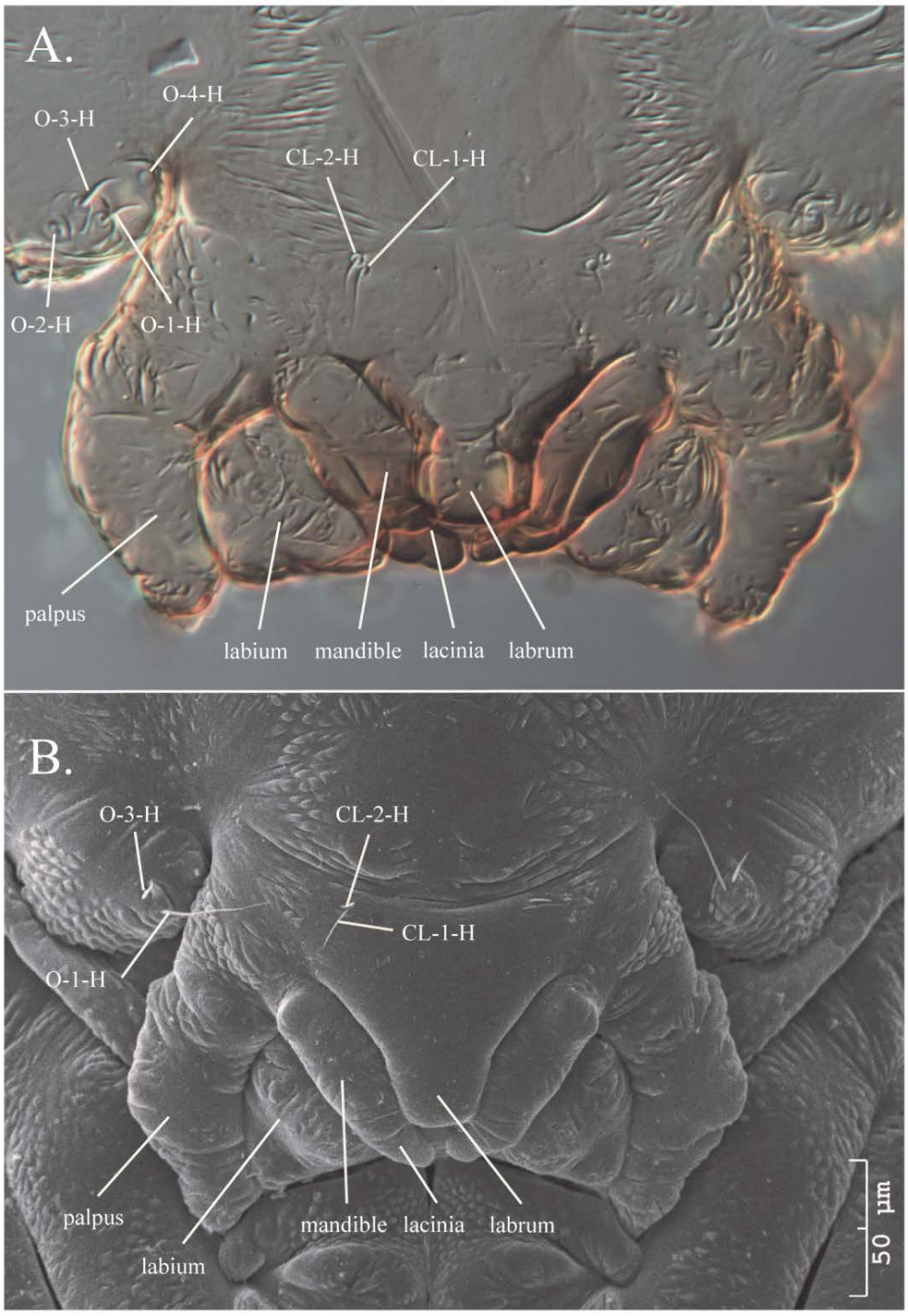

Fig. A-9. Structures and sensilla of the pupal mouth of *Culicoides sonorensis*. (A) With compound microscope. (B) With SEM.

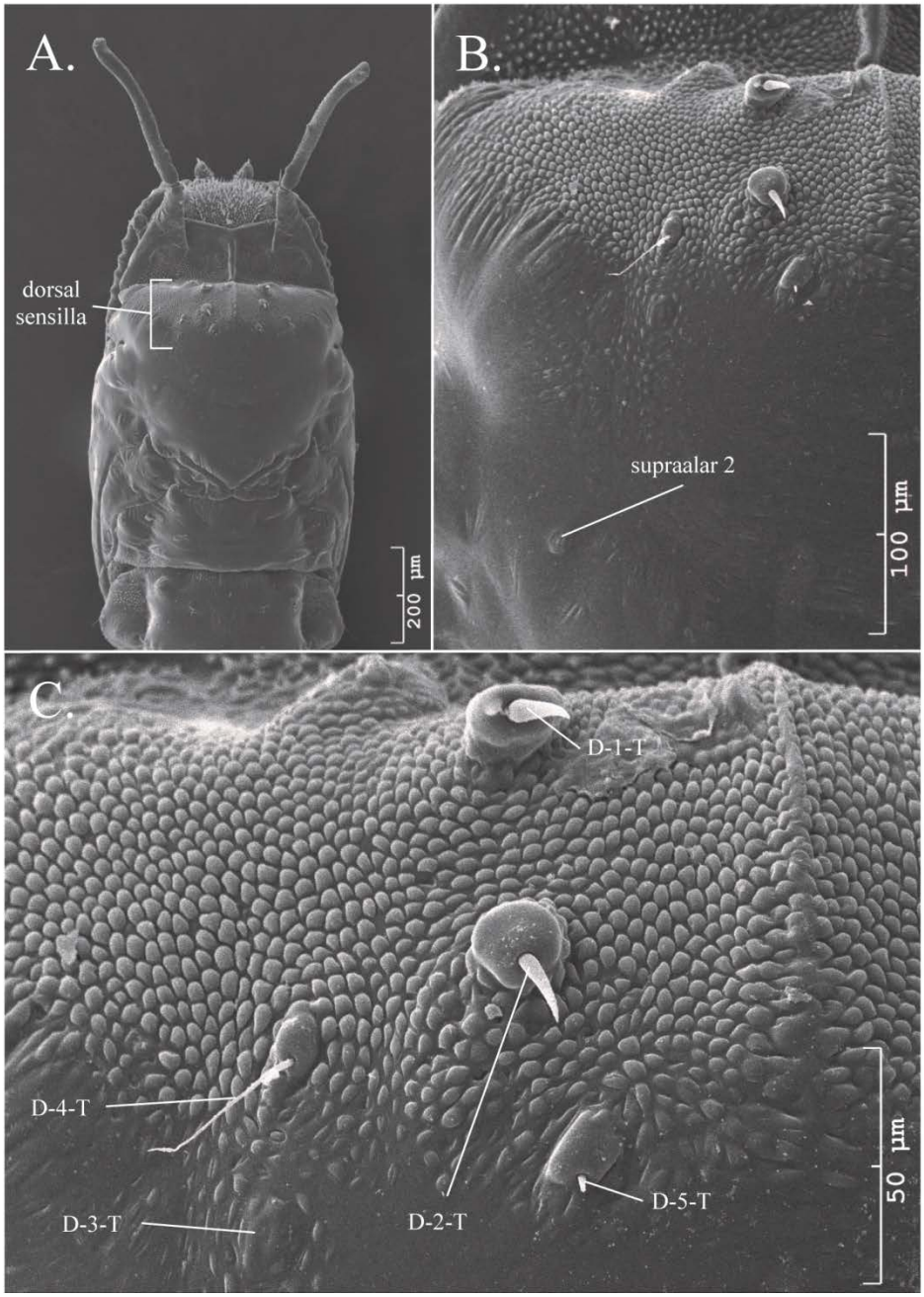

Fig. A-10. Structures and sensilla of the pupal thorax on *Culicoides sonorensis*, in dorsal view with SEM, increasing magnification A through *C.* (A) Entire thorax. (B) Dorsal sensilla, left side. (C) Dorsal sensilla, left side.

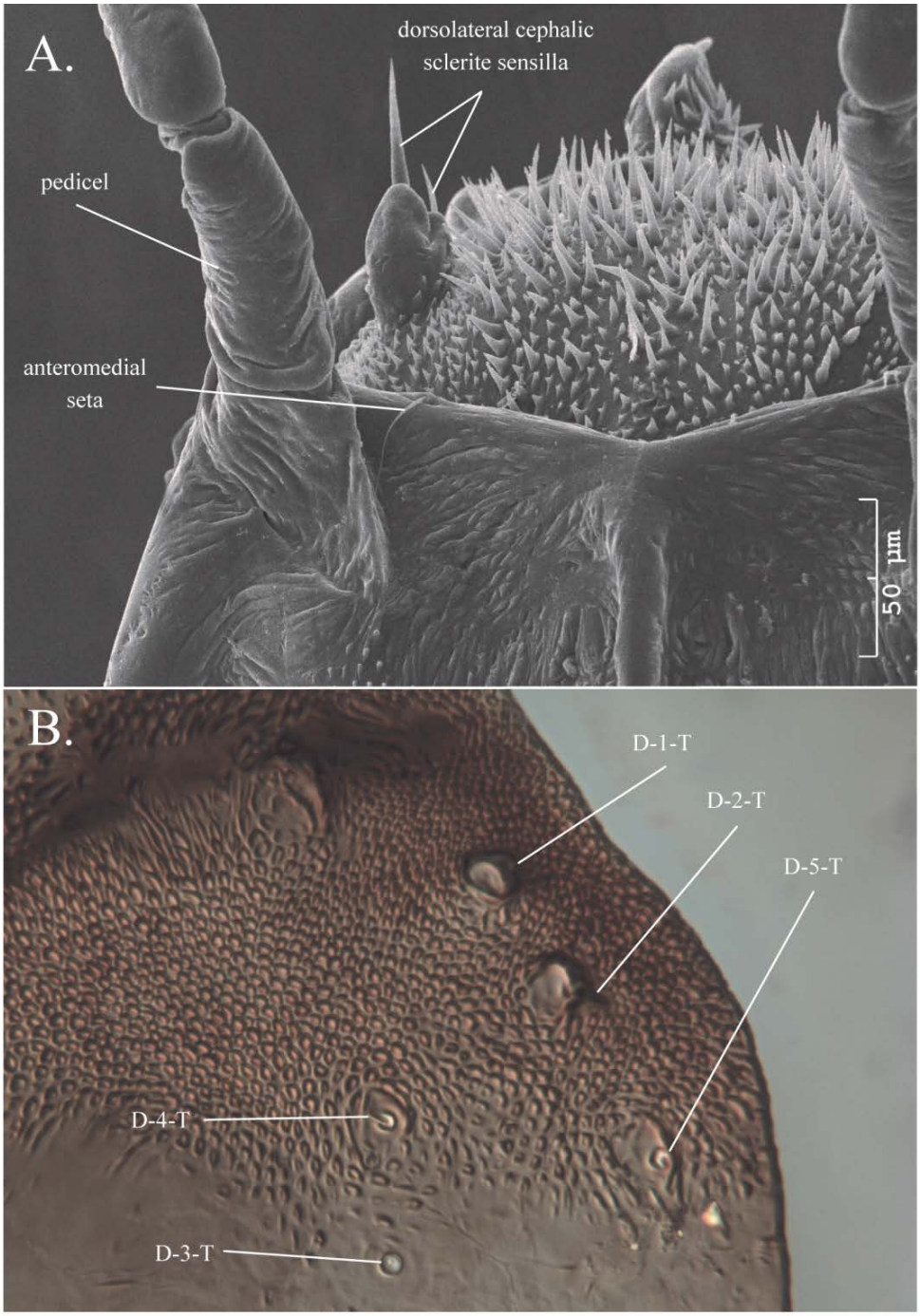

Fig. A-11. Structures of the pupal head and thorax of *Culicoides sonorensis* with SEM and compound microscope. (A) Pedicel and head in dorsal view, left side. (B) Dorsal seta, left side.

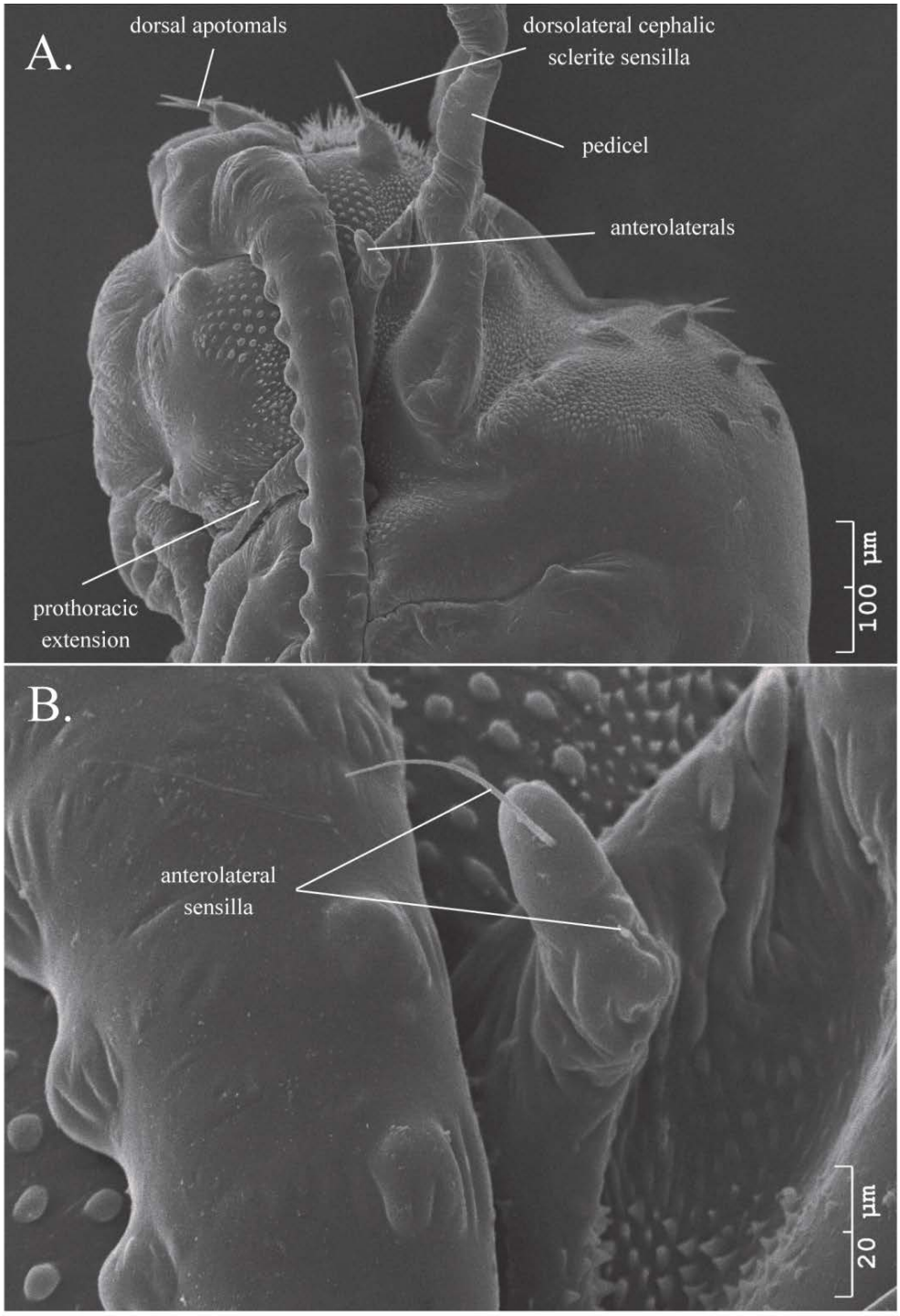

Fig. A-12. Structures of the pupal head and thorax of *Culicoides sonorensis*, left side with SEM. (A) Head and thorax. (B) Anterolateral sensilla.

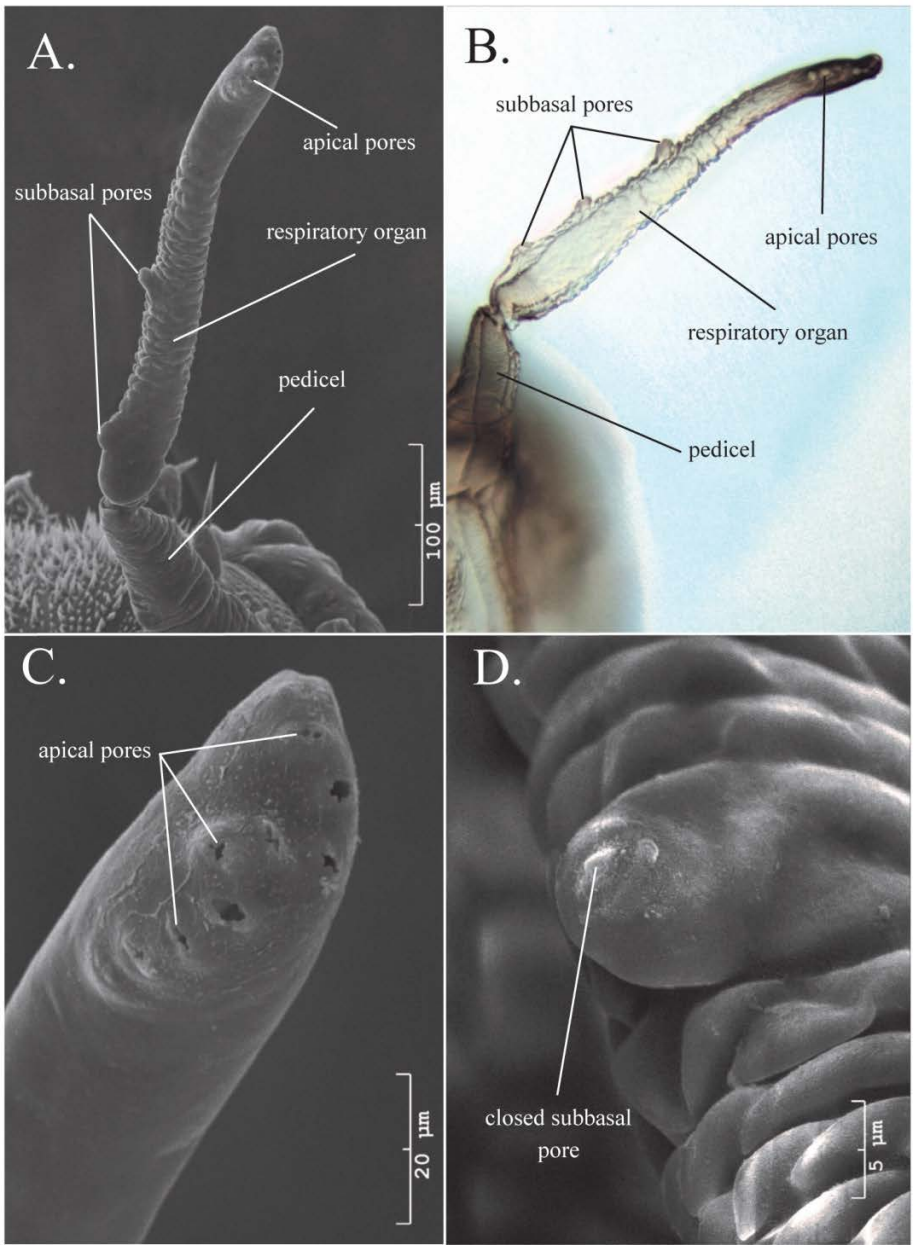

Fig. A-13. Structures of the pupal respiratory organ of *Culicoides sonorensis*. (A) Right pedicel and respiratory organ, in dorsal view with SEM. (B) Right pedicel and respiratory organ, in dorsal view with compound microscope. (C) Apex of respiratory organ with pores, in anterior view with SEM. (D) Subbasal pore, in dorsal view with SEM.

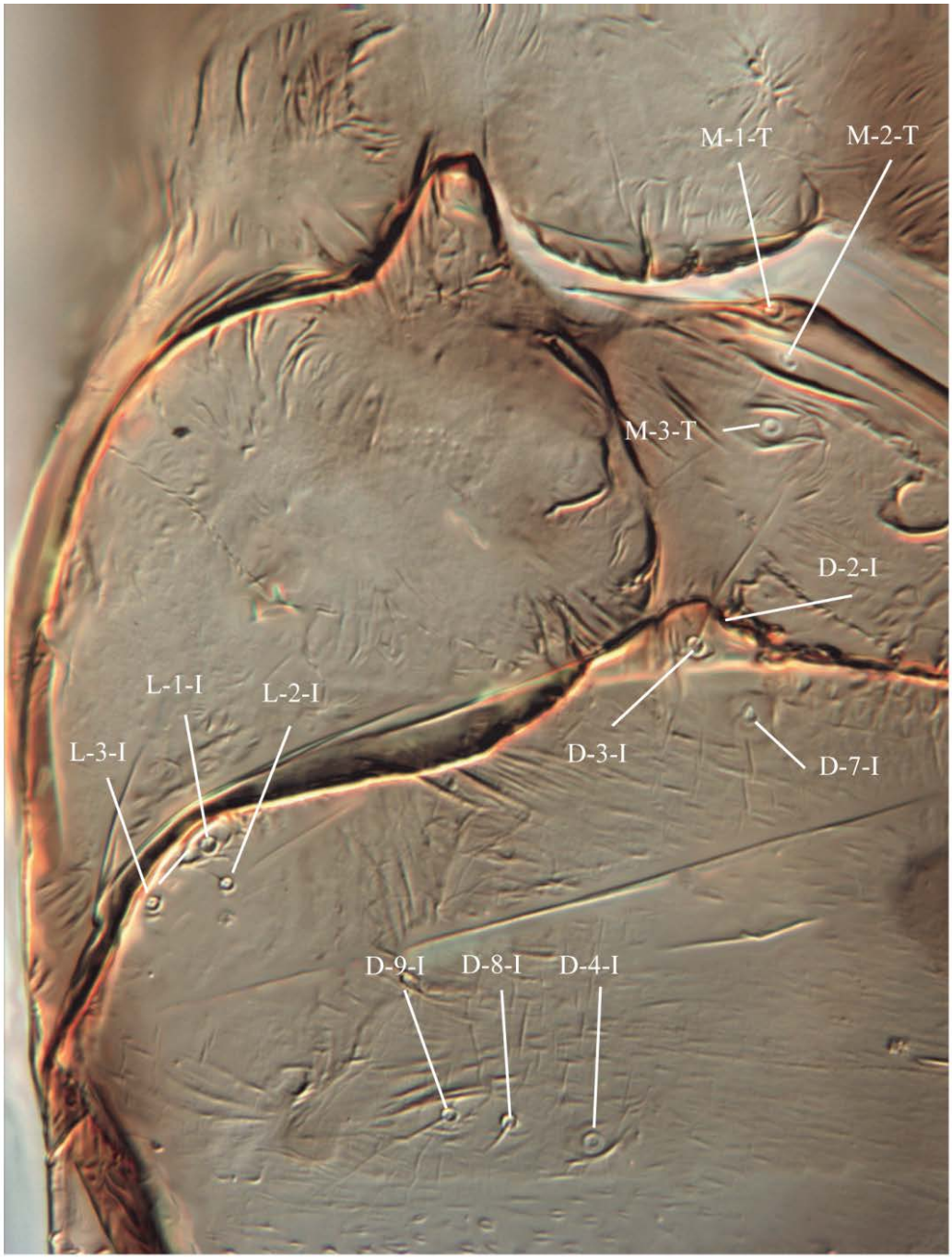

Fig. A-14. Structures and sensilla of the pupal metathorax and tergite 1 of *Culicoides sonorensis*, left side, in dorsal view with compound microscope.

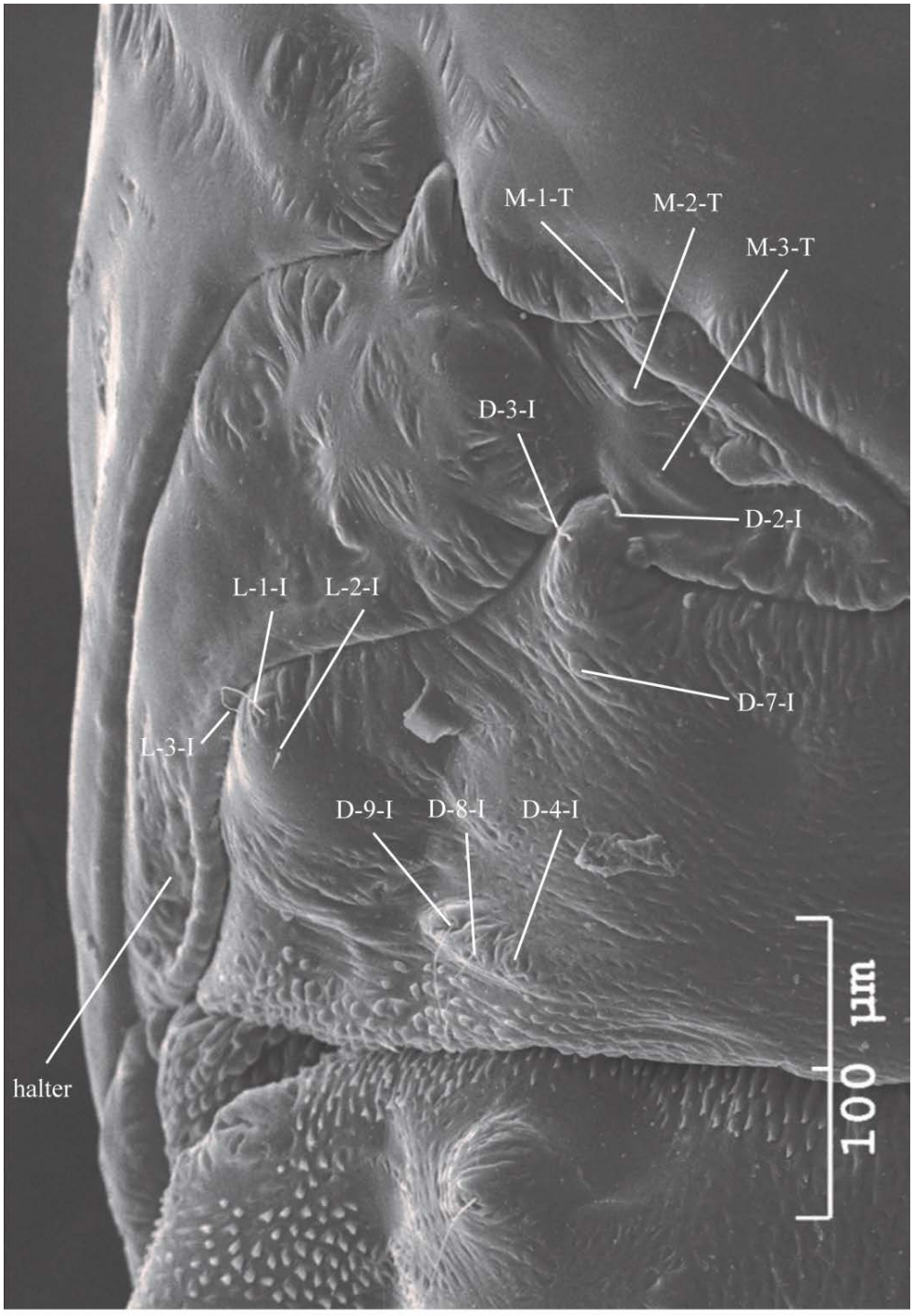

Fig 15. Structures and sensilla of the pupal metathorax and tergite 1 of *Culicoides sonorensis*, left side, in dorsal view with SEM.

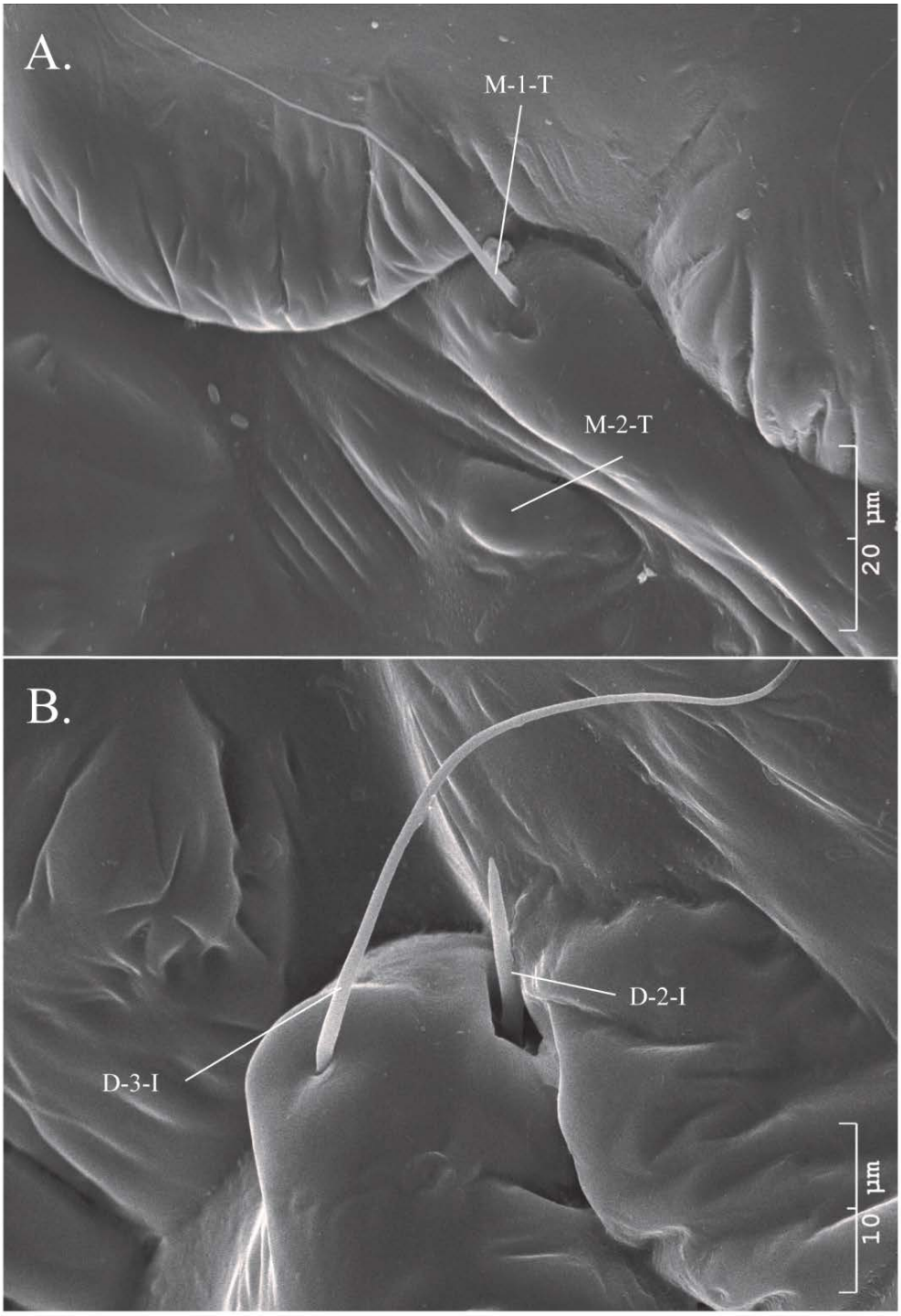

Fig. A-16. Structures of the pupal metathorax and tergite 1 of *Culicoides sonorensis*, left side with SEM. See Fig. A-15 for context. (A) M-1-T, M-2-T. (B) D-2-I, D-3-I.

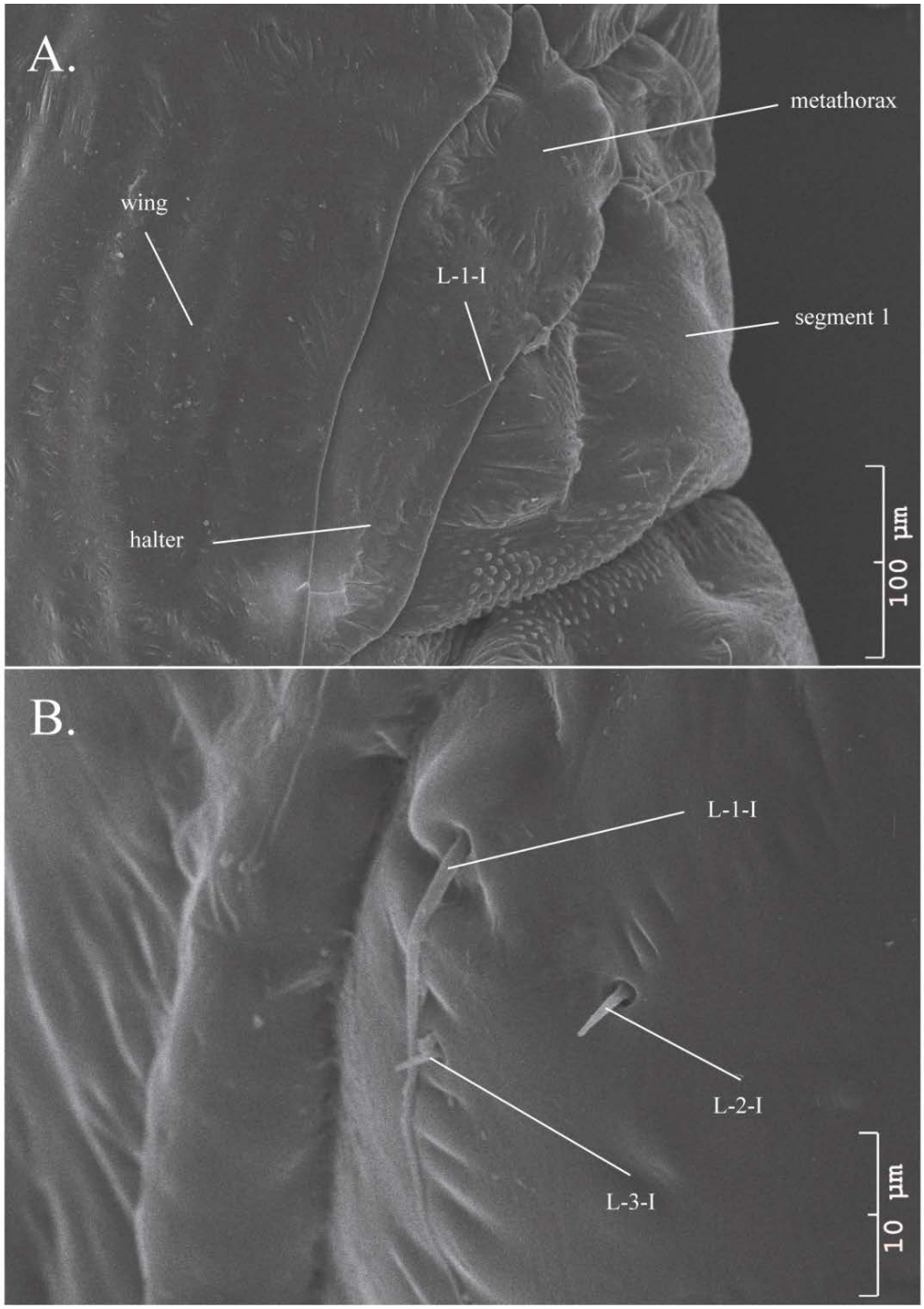

Fig. A-17. Structures and sensilla of the pupal metathorax and abdomen of *Culicoides sonorensis*, left side with SEM. (A) Metathorax and segment 1. (B) Lateral sensilla, segment 1.

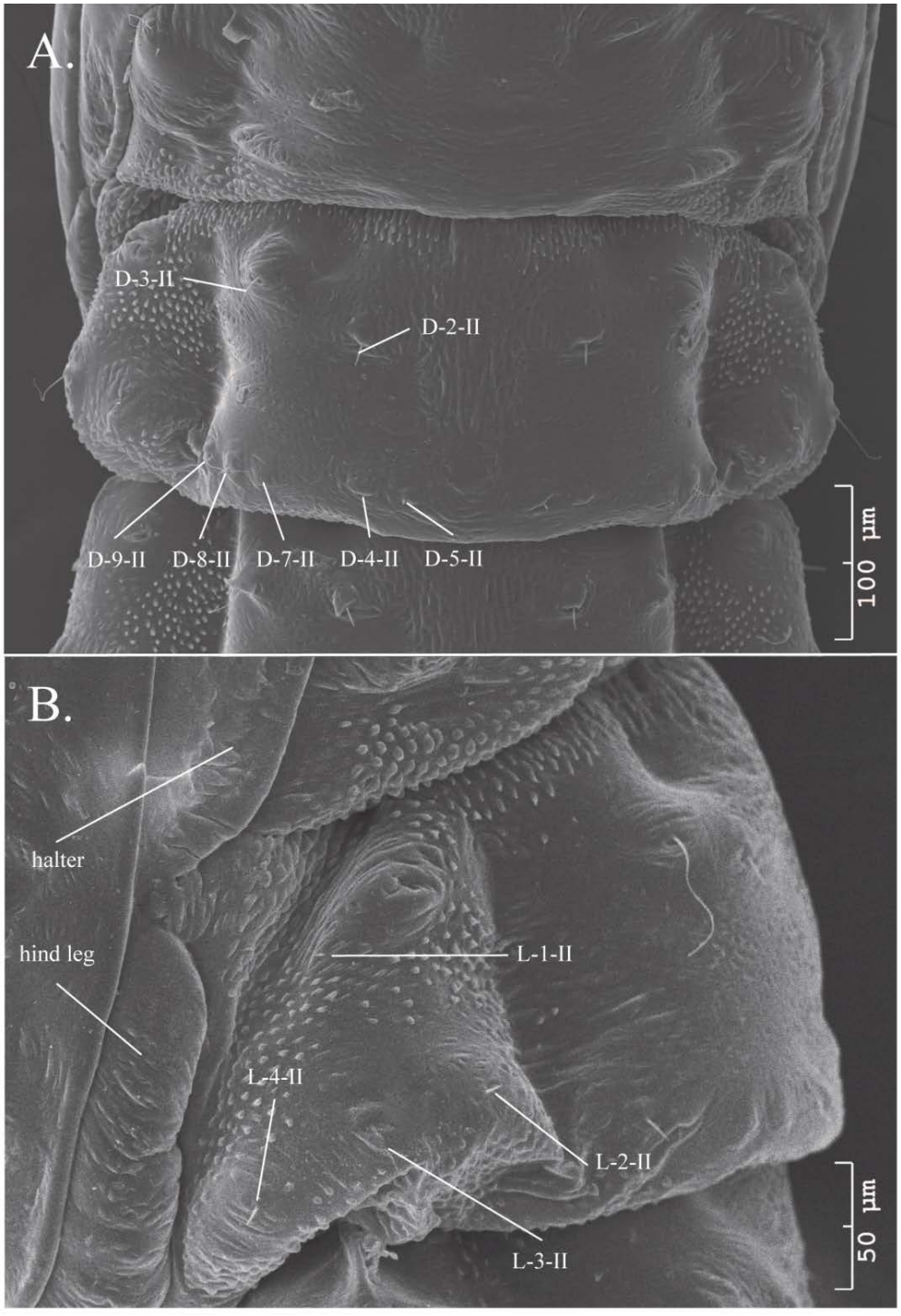

Fig. A-18. Structures and sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Tergite 2. (B) Segment 2, left side.

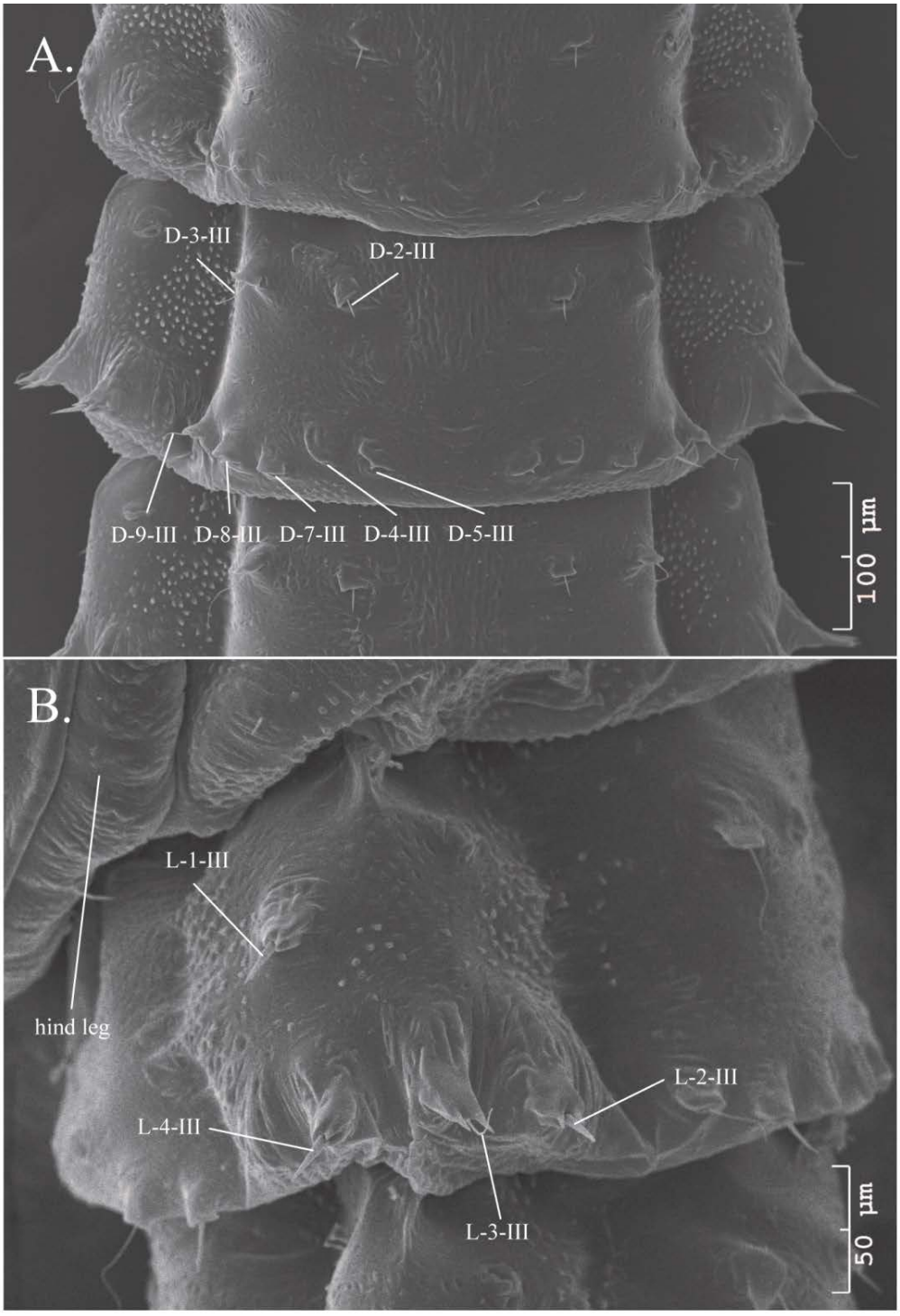

Fig. A-19. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Tergite 3. (B) Segment 3, left side.

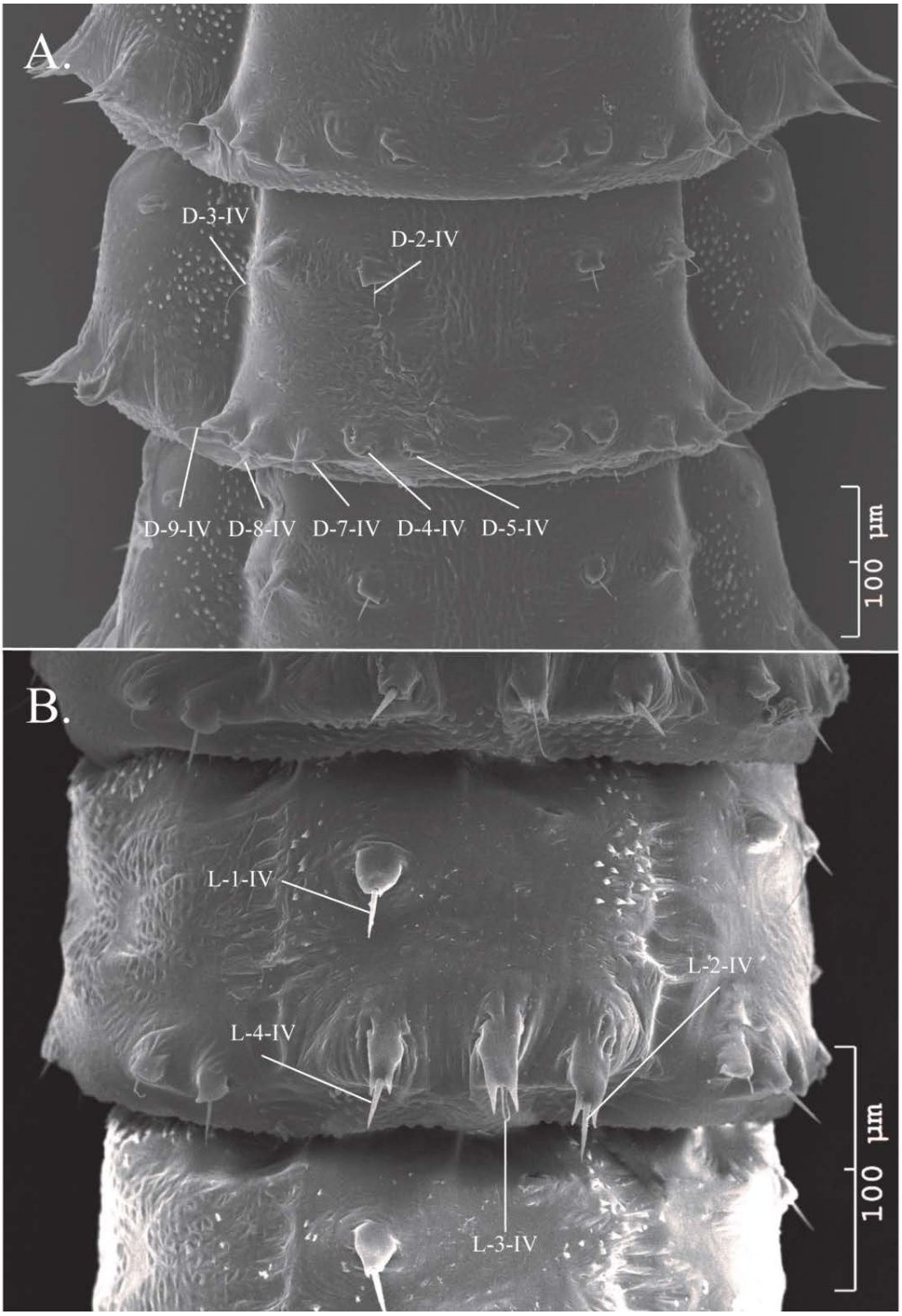

Fig. A-20. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Tergite 4. (B) Segment 4, left side.

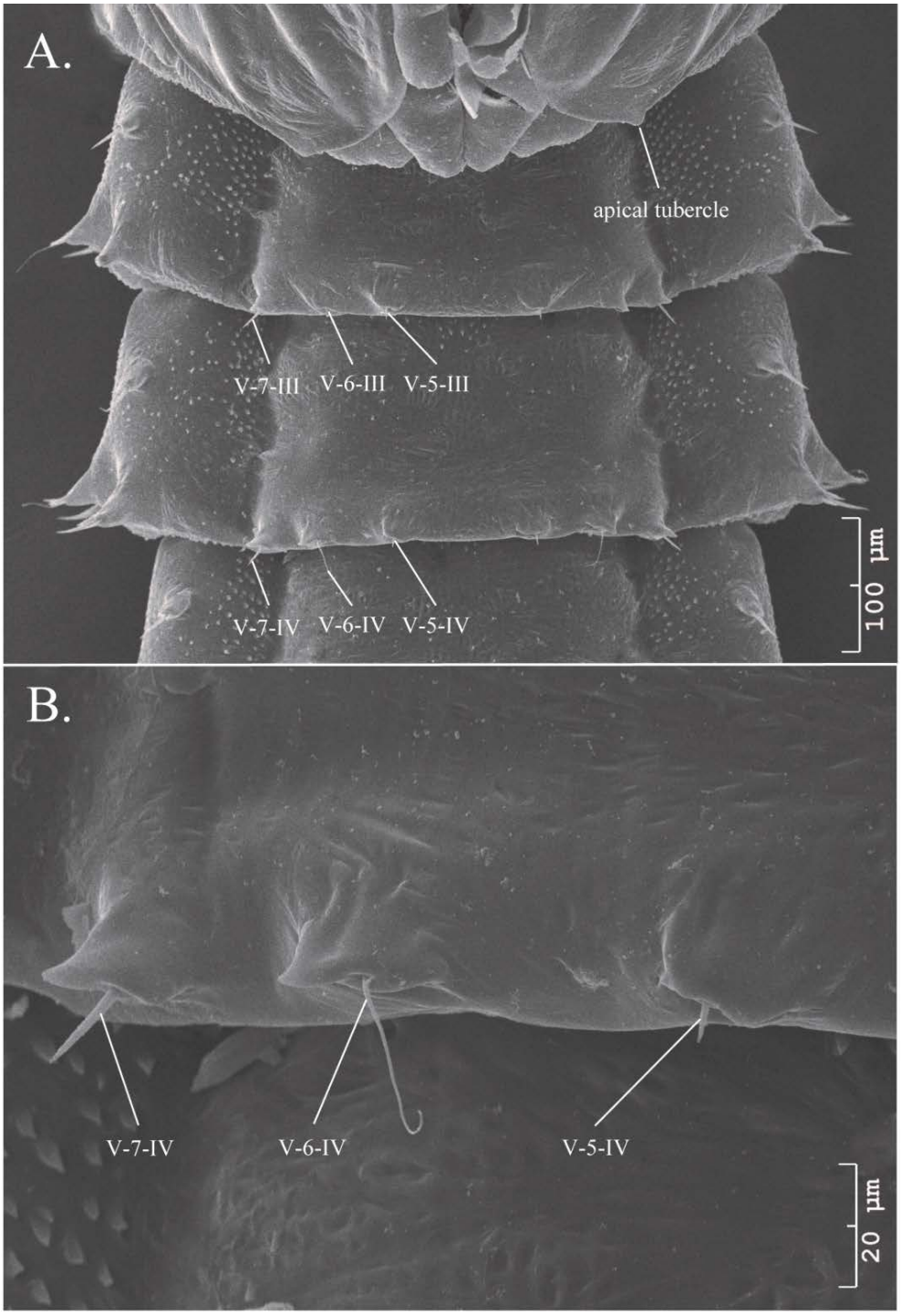

Fig. A-21. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Sternite 4. (B) V-5-IV, V-6-IV, V-7-IV.

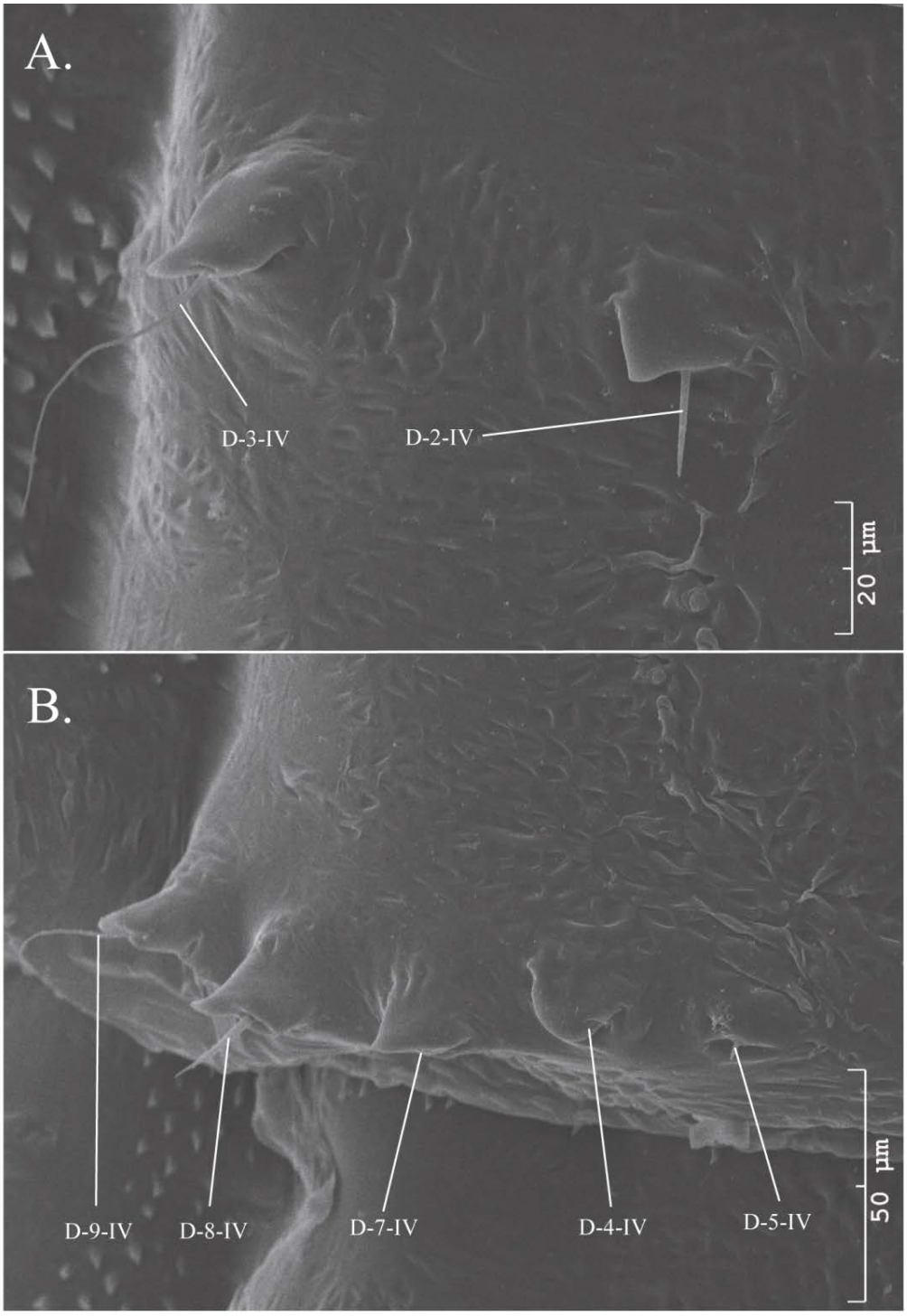

Fig. A-22. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM, see Fig. A-20 for context. (A) D-2-IV, D-3-IV. (B) D-5-IV, D-6-IV, D-7-IV, D-8-IV, D-9-IV.

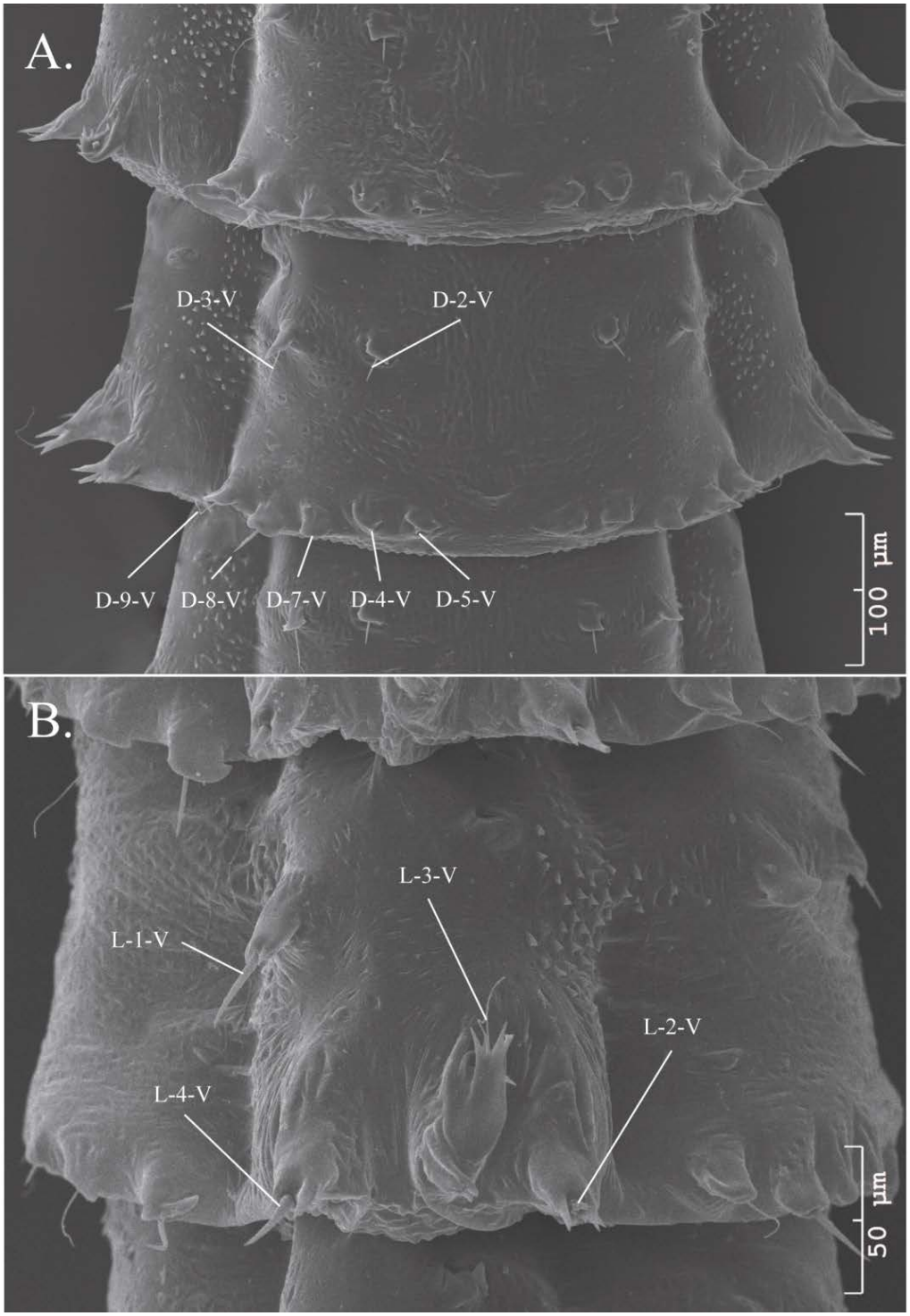

Fig. A-23. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Tergite 5. (B) Segment 5, left side.

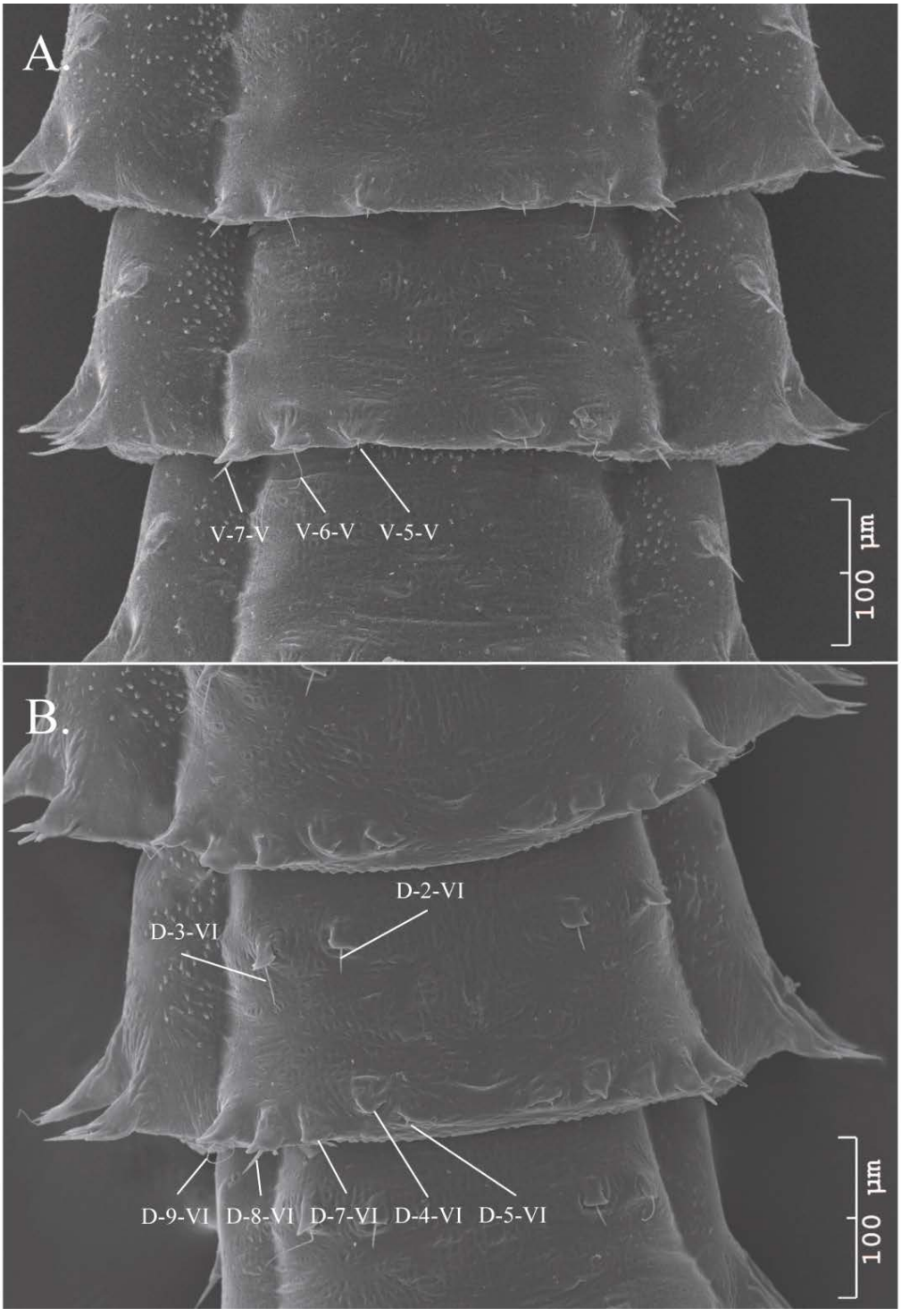

Fig. A-24. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Sternite 5. (B) Tergite 6.

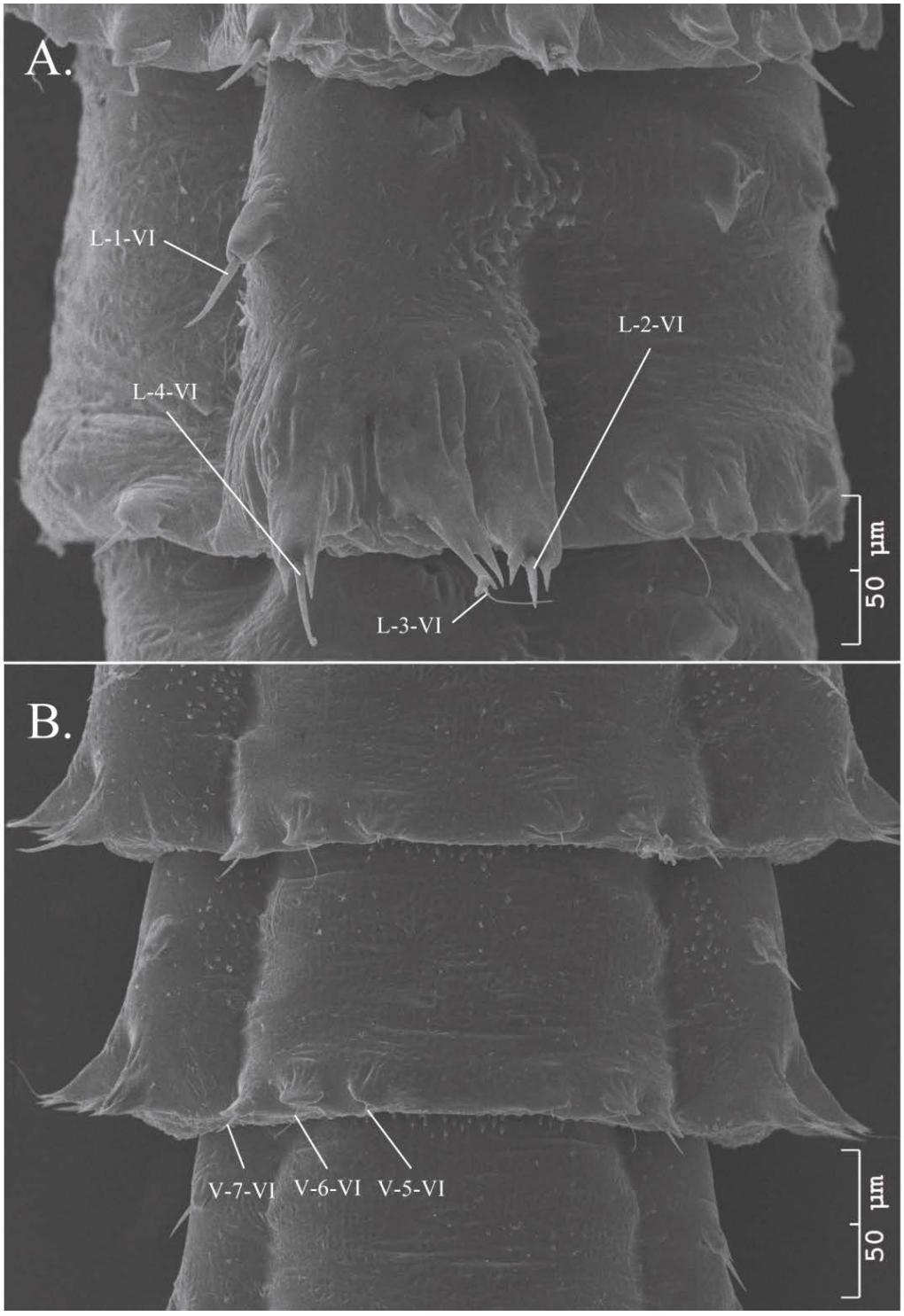

Fig. A-25. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) segment 6, left side. (B) Sternite 6.

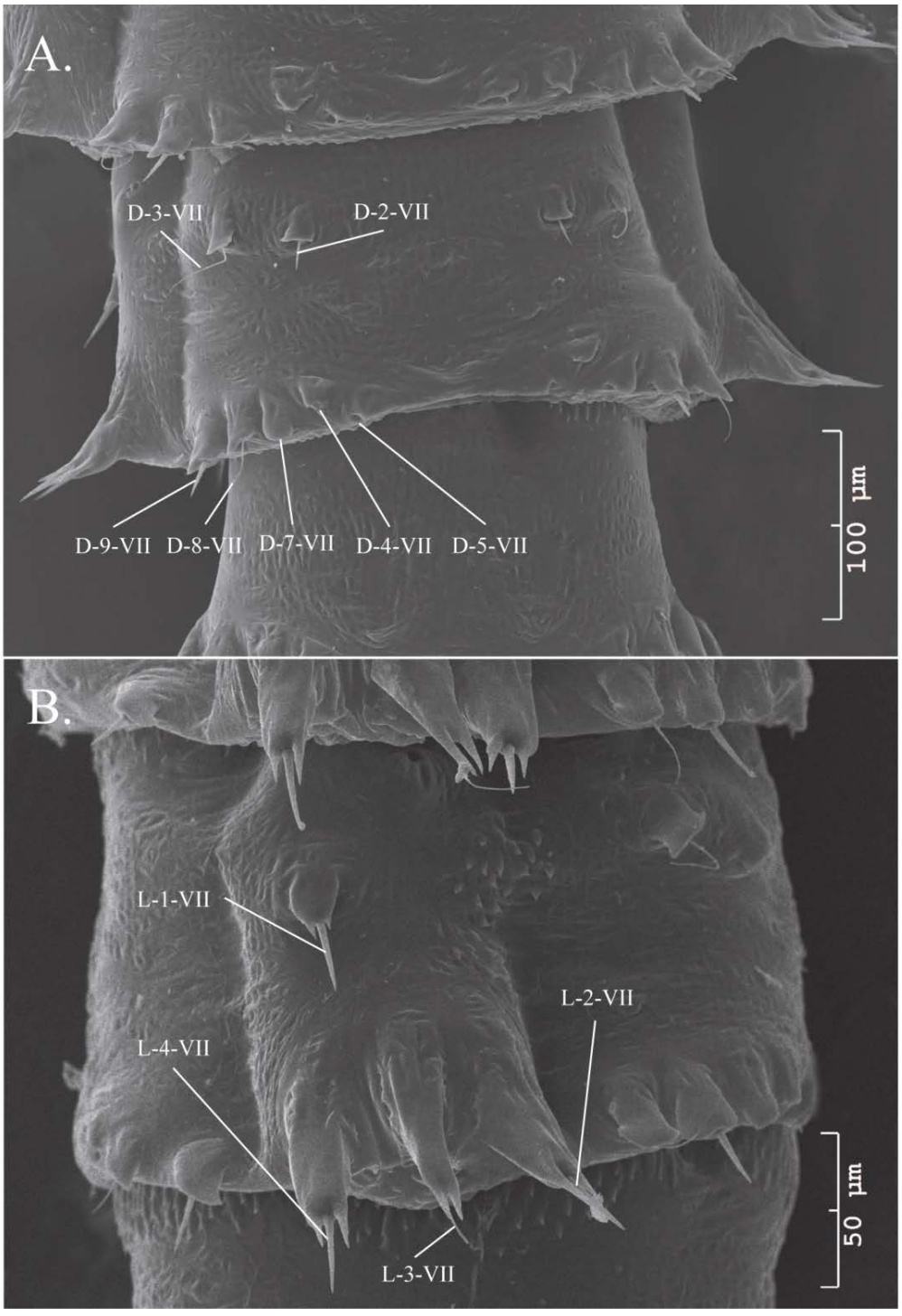

Fig. A-26. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Tergite 7. (B) Segment 7, left side.

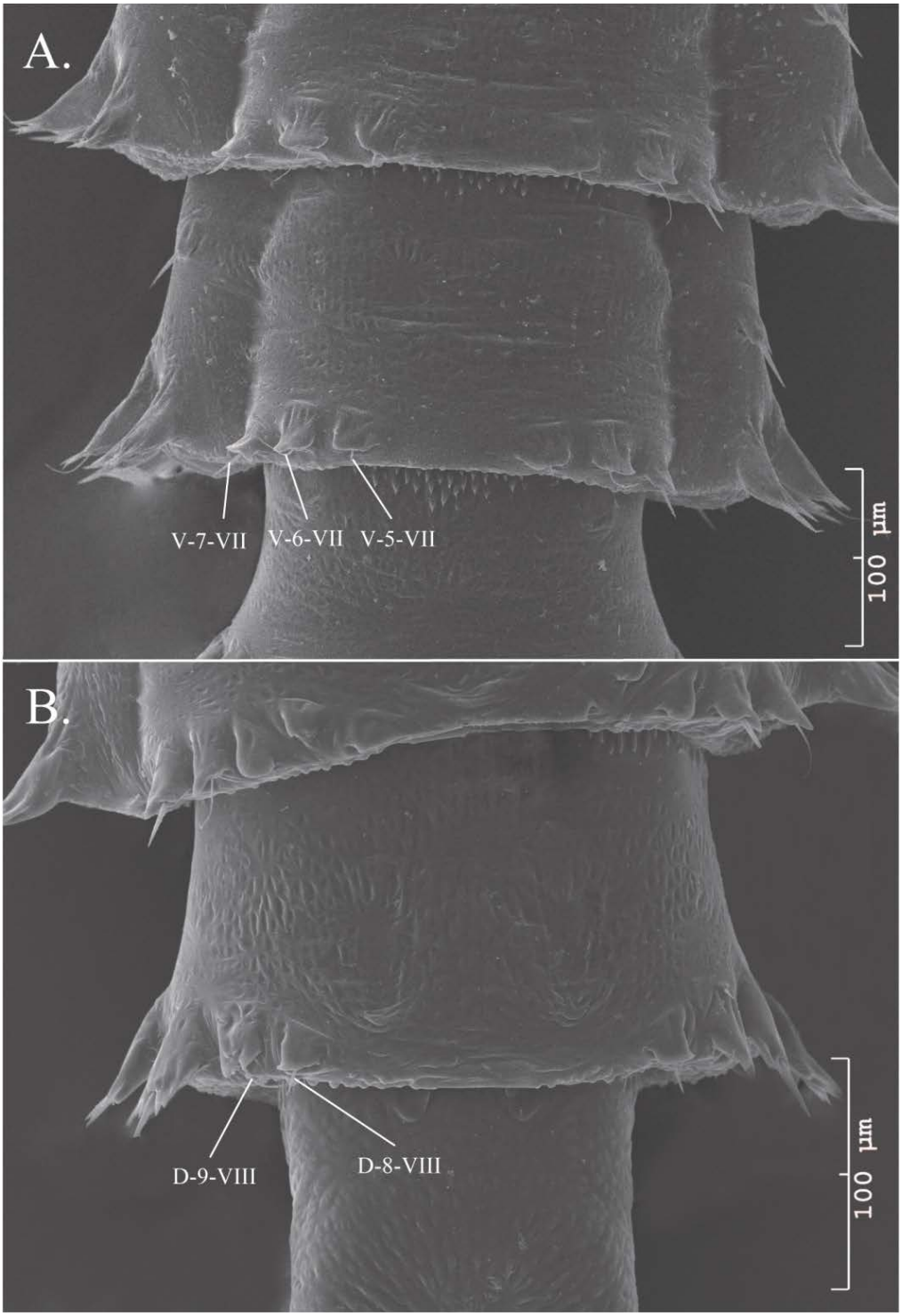

Fig. A-27. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Segment 7, left side. (B) Tergite 8.

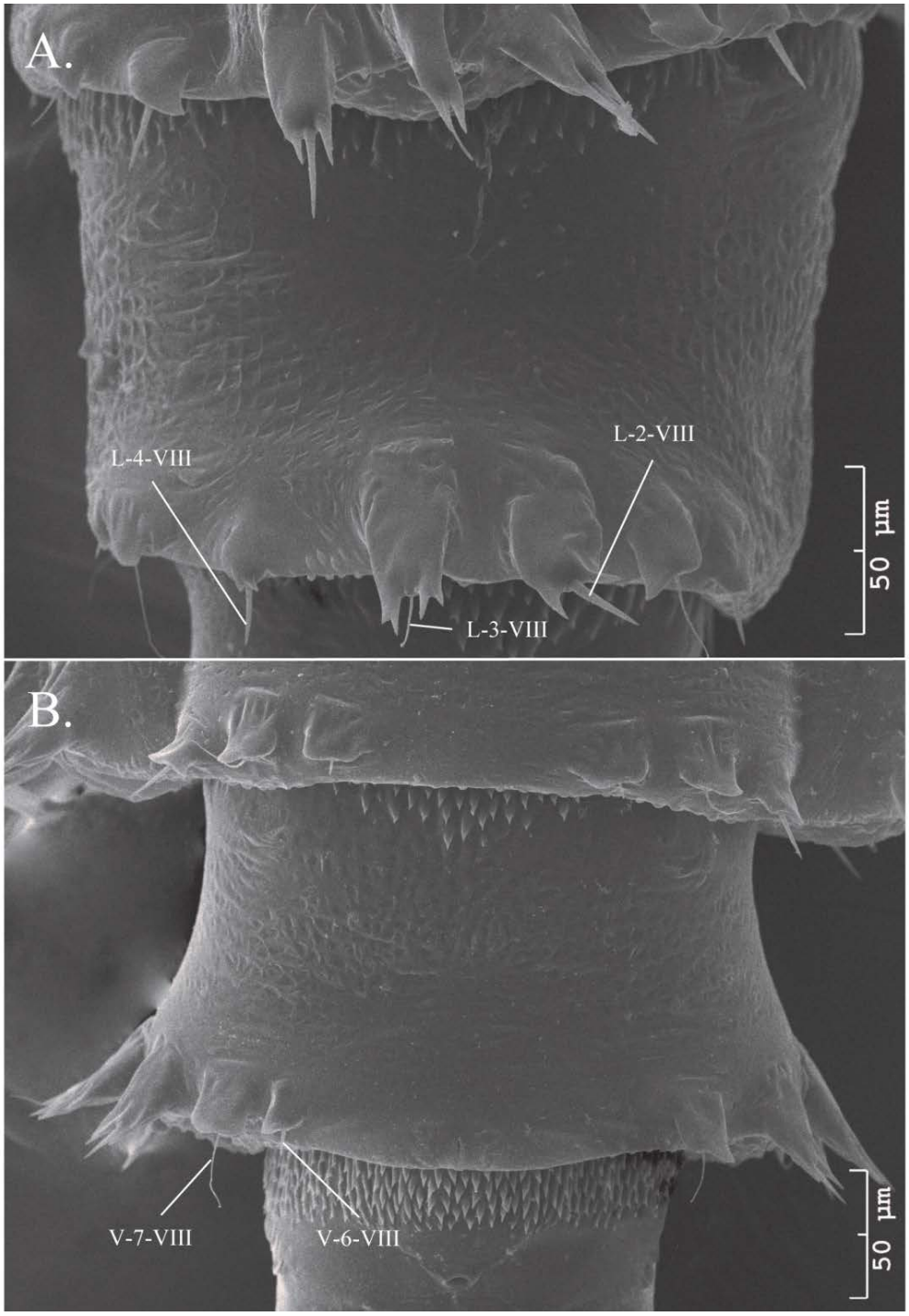

Fig. A-28. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Segment 8, left side. (B) Sternite 8.

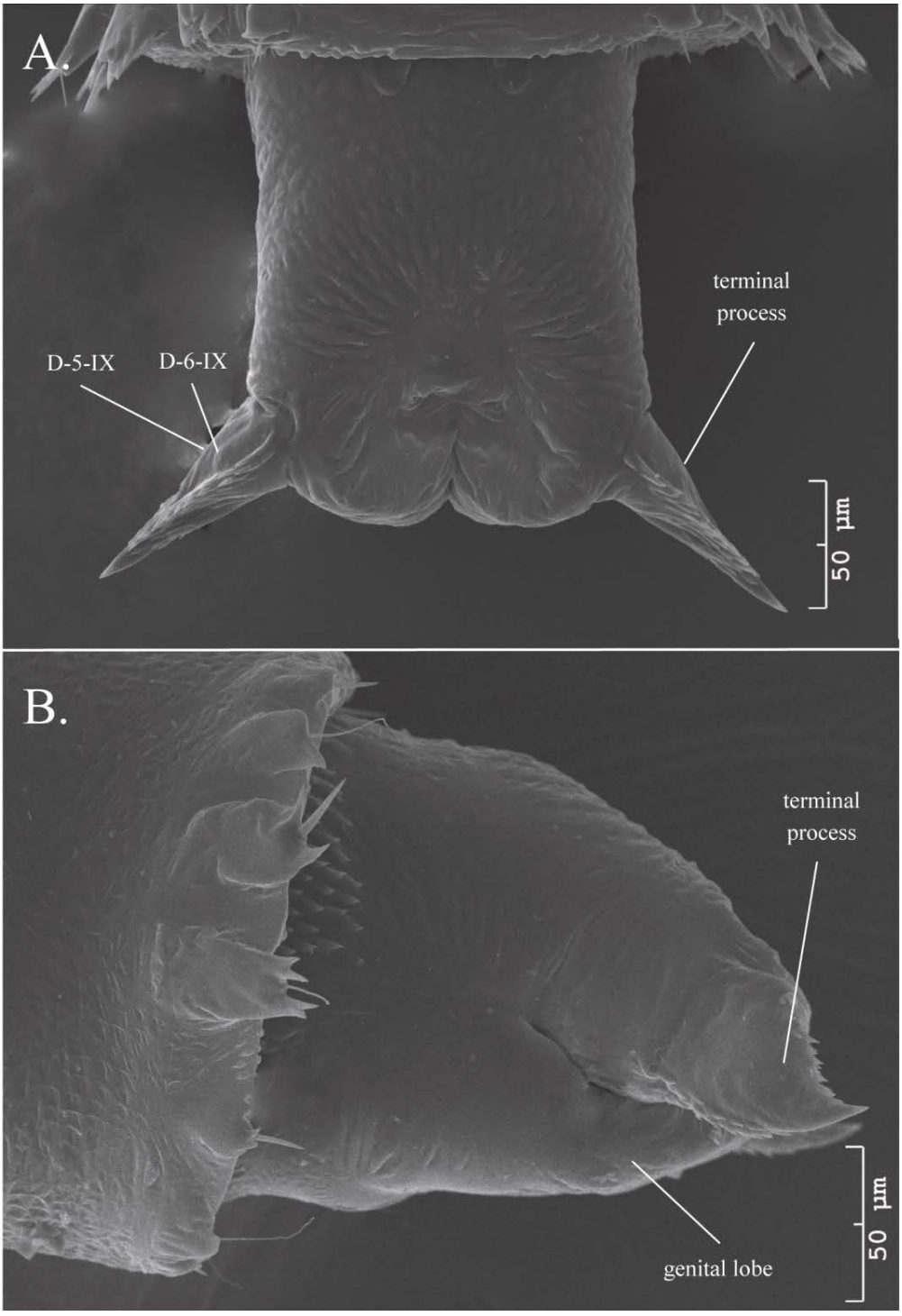

Fig. A-29. Sensilla of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Female, tergite 9. (B) Male, segment 9, left side.

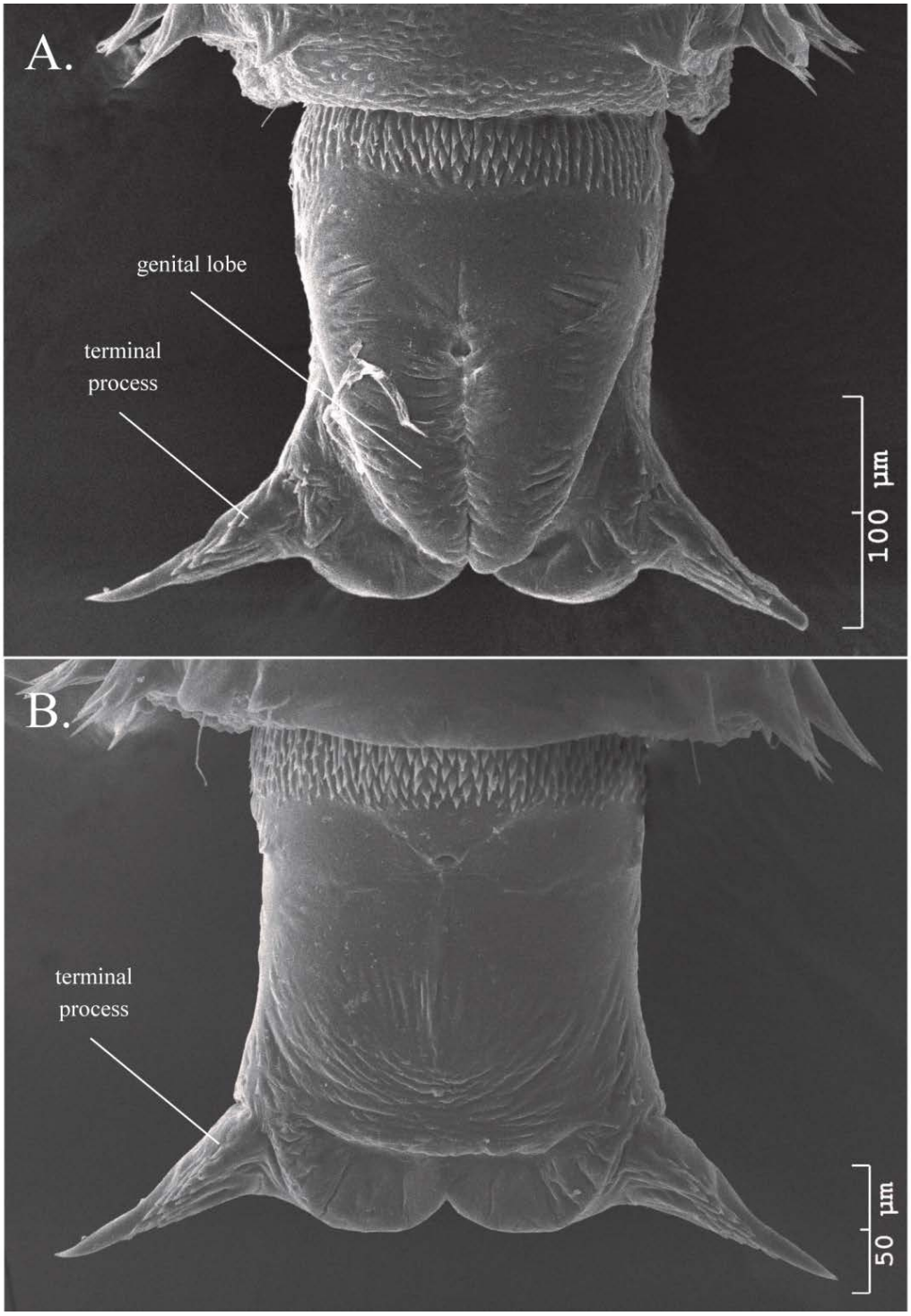

Fig. A-30. Structures of the pupal abdomen of *Culicoides sonorensis* with SEM. (A) Male, sternite 9. (B) Female, sternite 9.

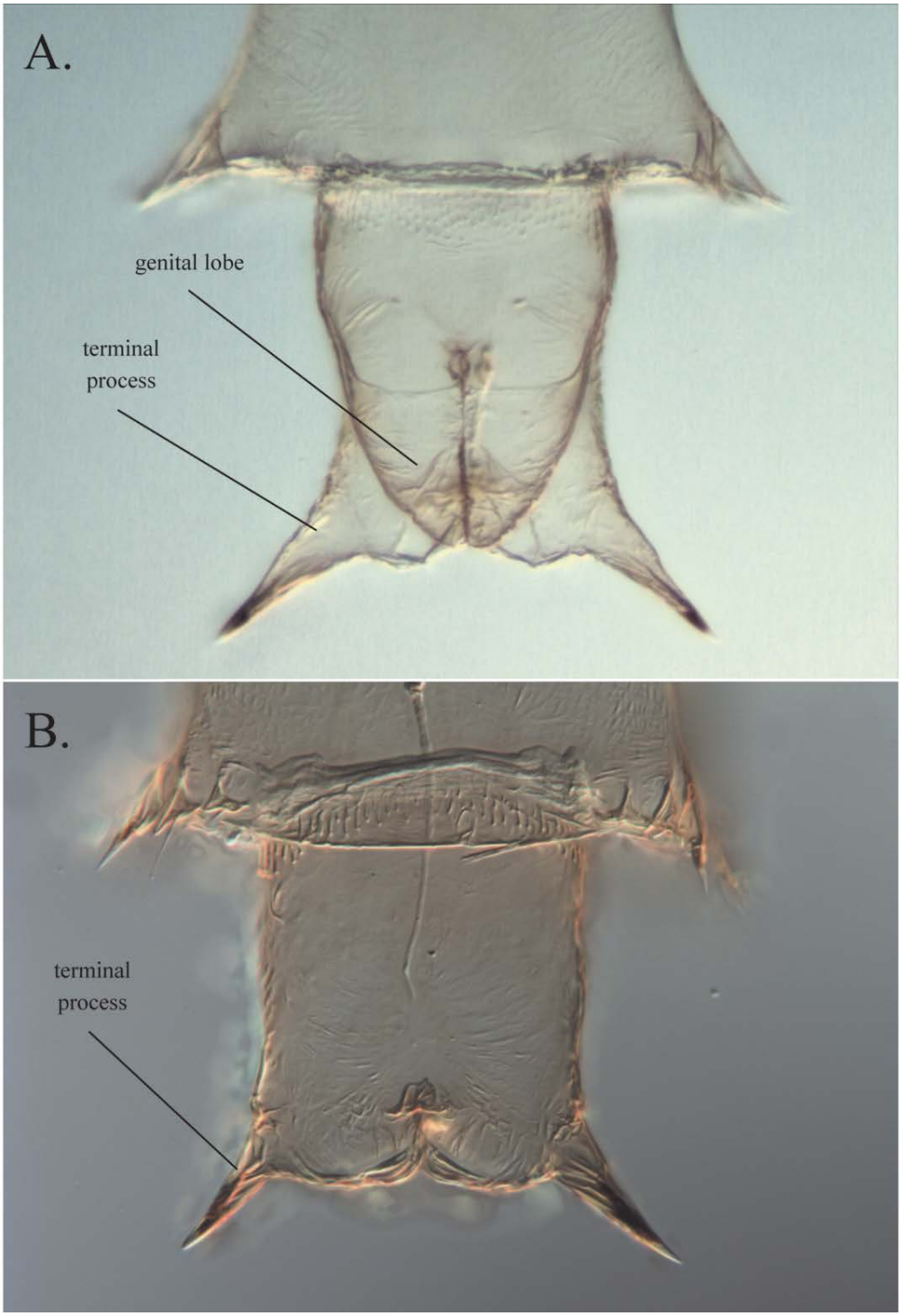

Fig. A-31. Structures of the pupal abdomen of *Culicoides sonorensis* with compound microscope. (A) Male, sternite 9. (B) Female, sternite 9.

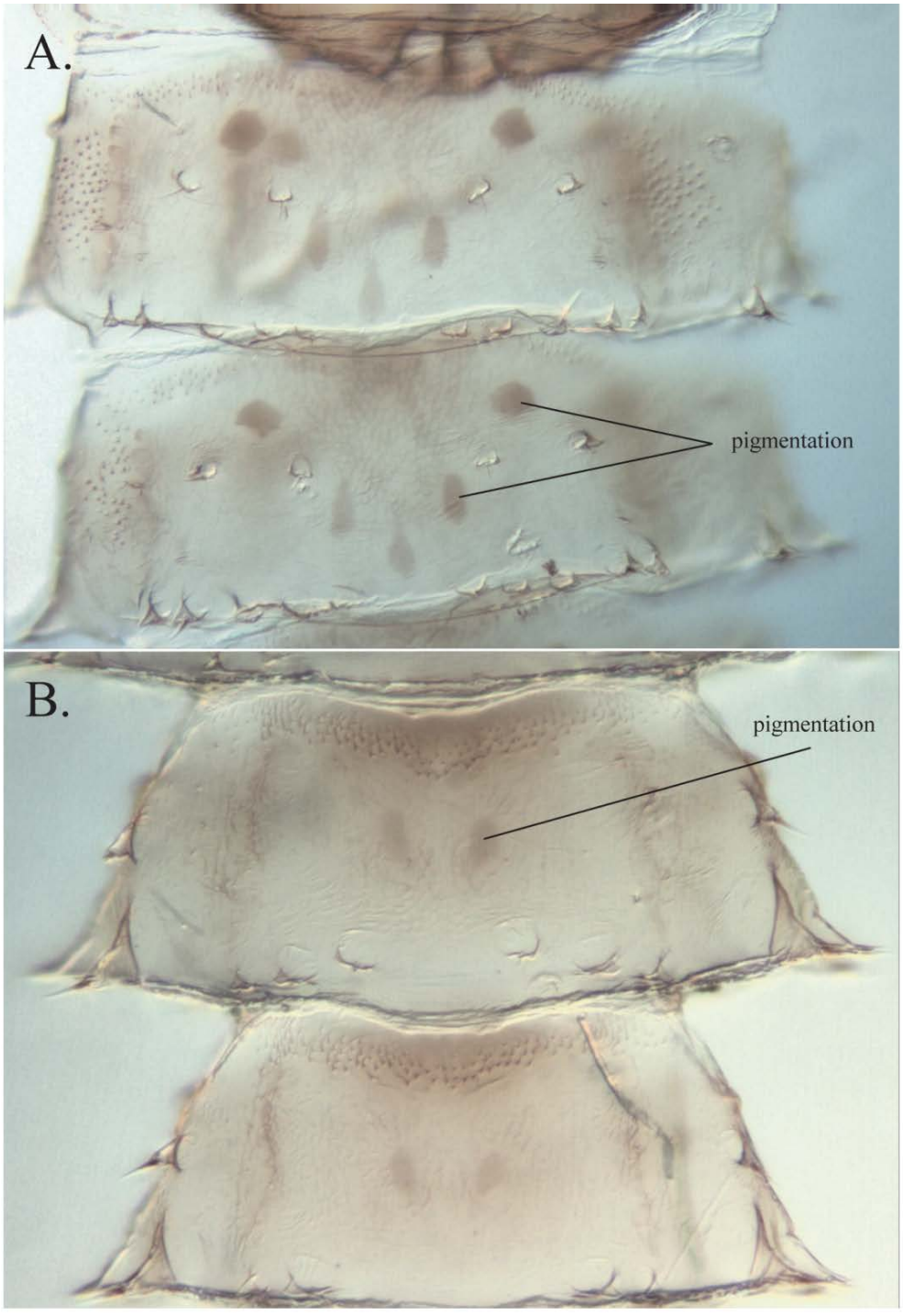

Fig. A-32. Sensilla of the pupal abdomen of *Culicoides sonorensis* with compound microscope. (A) Tergites 3 and 4. (B) Sternite 4 and 5.

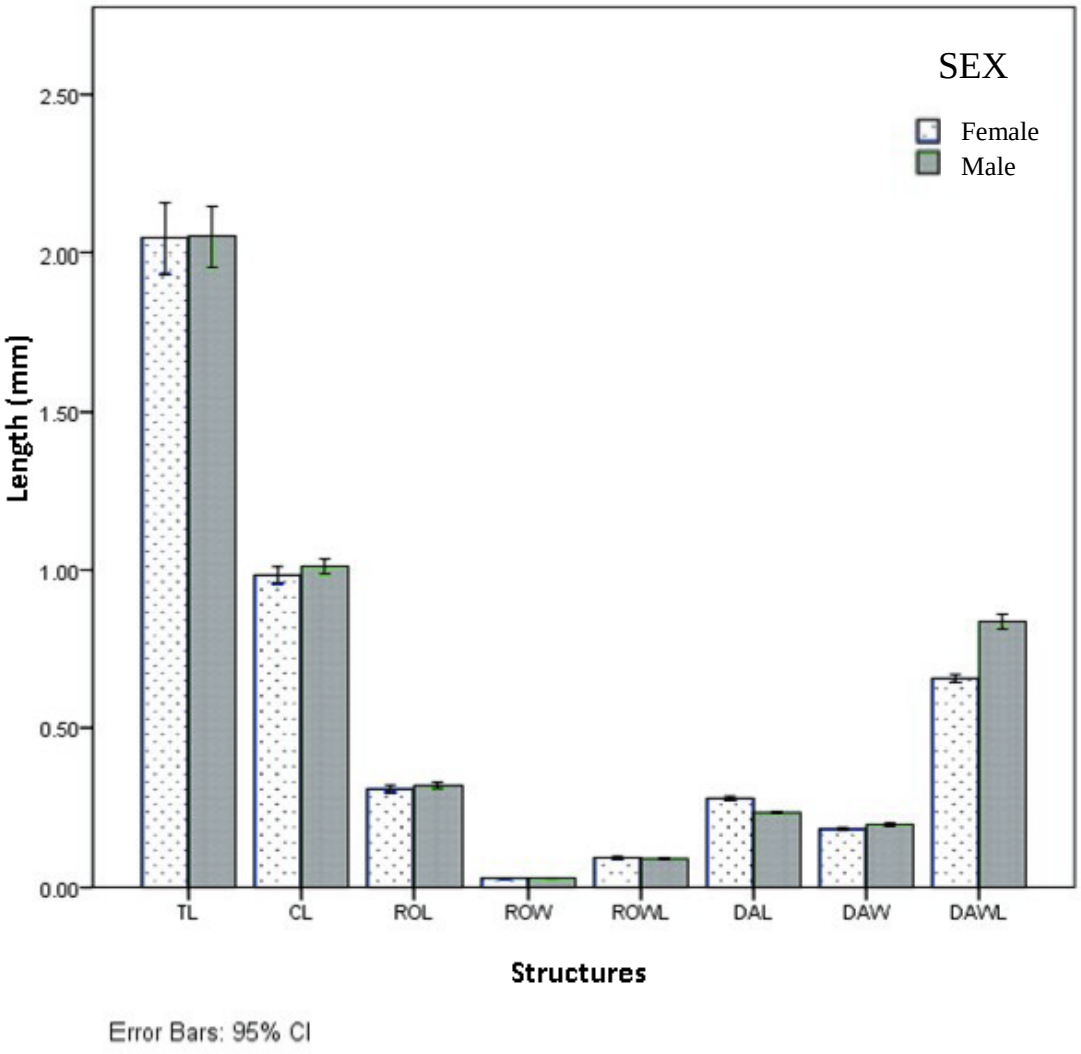

Fig. A-33. Measurements and ratios of male and female pupae of *C. sonorensis* (mm). Total length (TL), cephalothorax length (CL), respiratory organ length (ROL), respiratory organ width (ROW), respiratory organ ratio W/L (ROWL), dorsal apotome length (DAL), dorsal apotome width (DAW), dorsal apotome ratio W/L (DAWL).

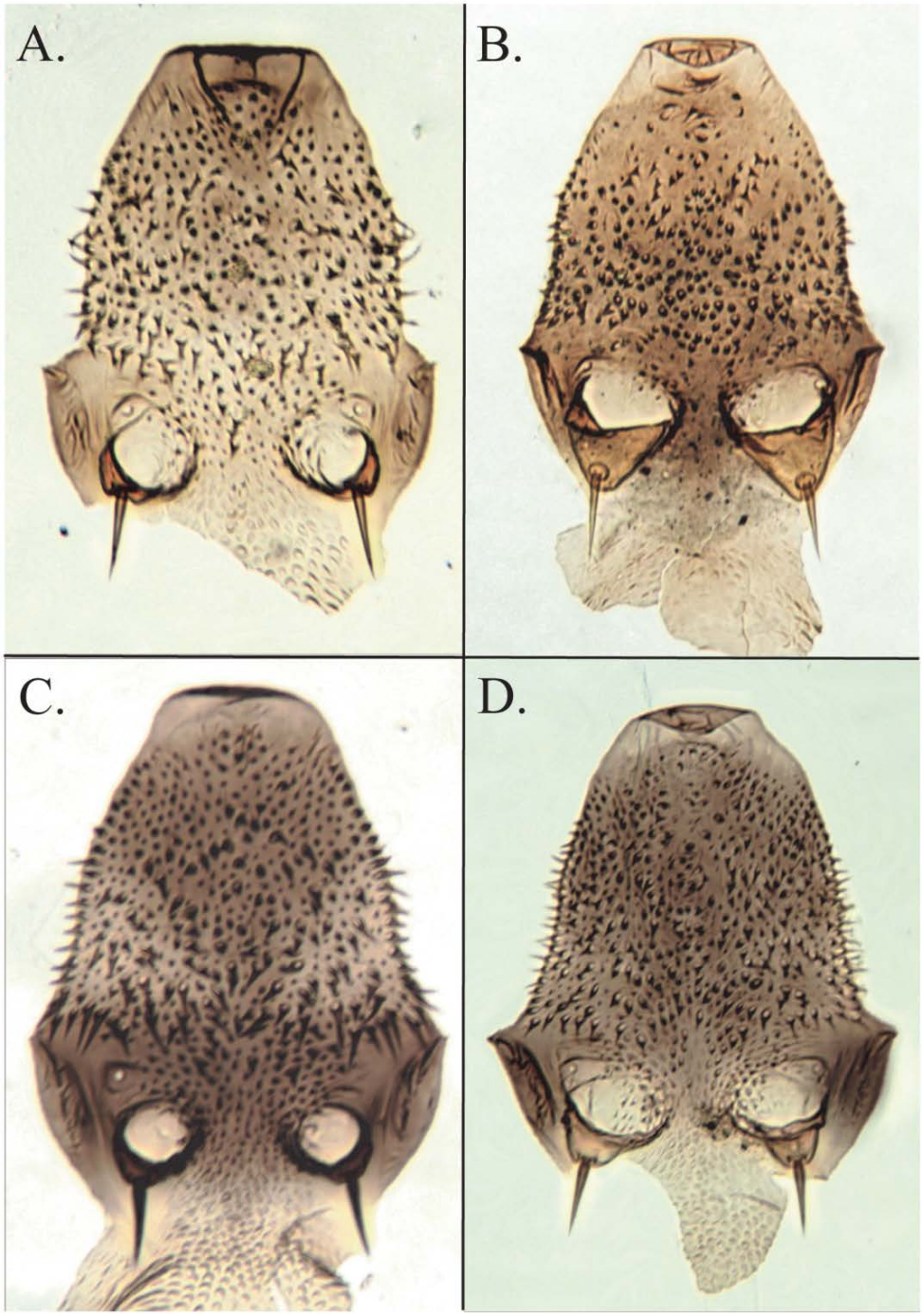

Fig. A-34. Female, dorsal apotome, in anterior view with compound light microscope. (A) *Culicoides nubeculosus*. (B) *Culicoides occidentalis*. (C) *Culicoides sonorensis*. (D) *Culicoides variipennis*.

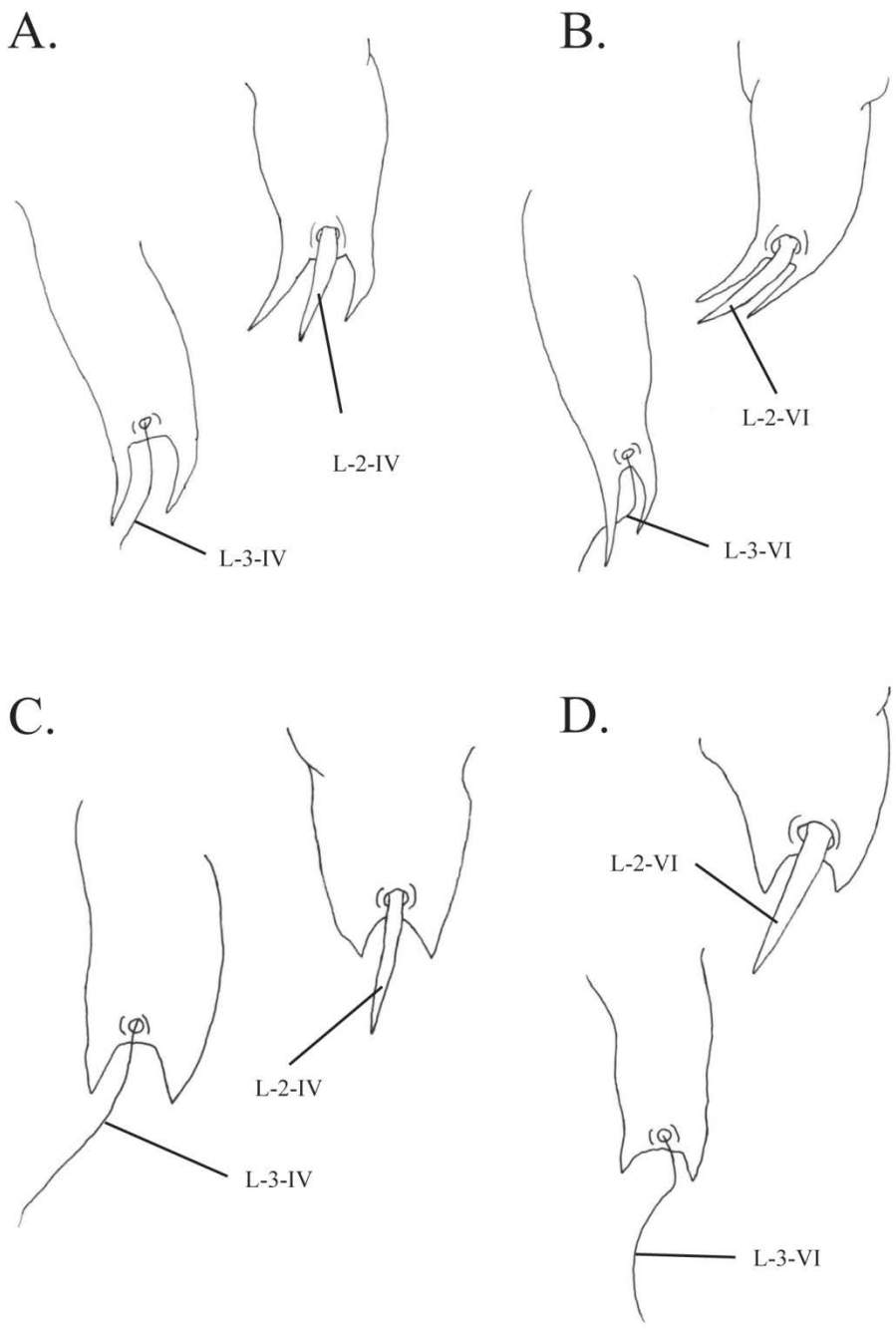

Fig. A-35. Male, left lateral sensilla in dorsal view. (A) L-3-IV and L-2-IV of *Culicoides sonorensis*. (B) L-3-VI and L-2-VI of *Culicoides sonorensis*. (C) L-3-IV and L-2-IV of *Culicoides variipennis*. (D) L-3-VI and L-2-VI of *Culicoides variipennis*.

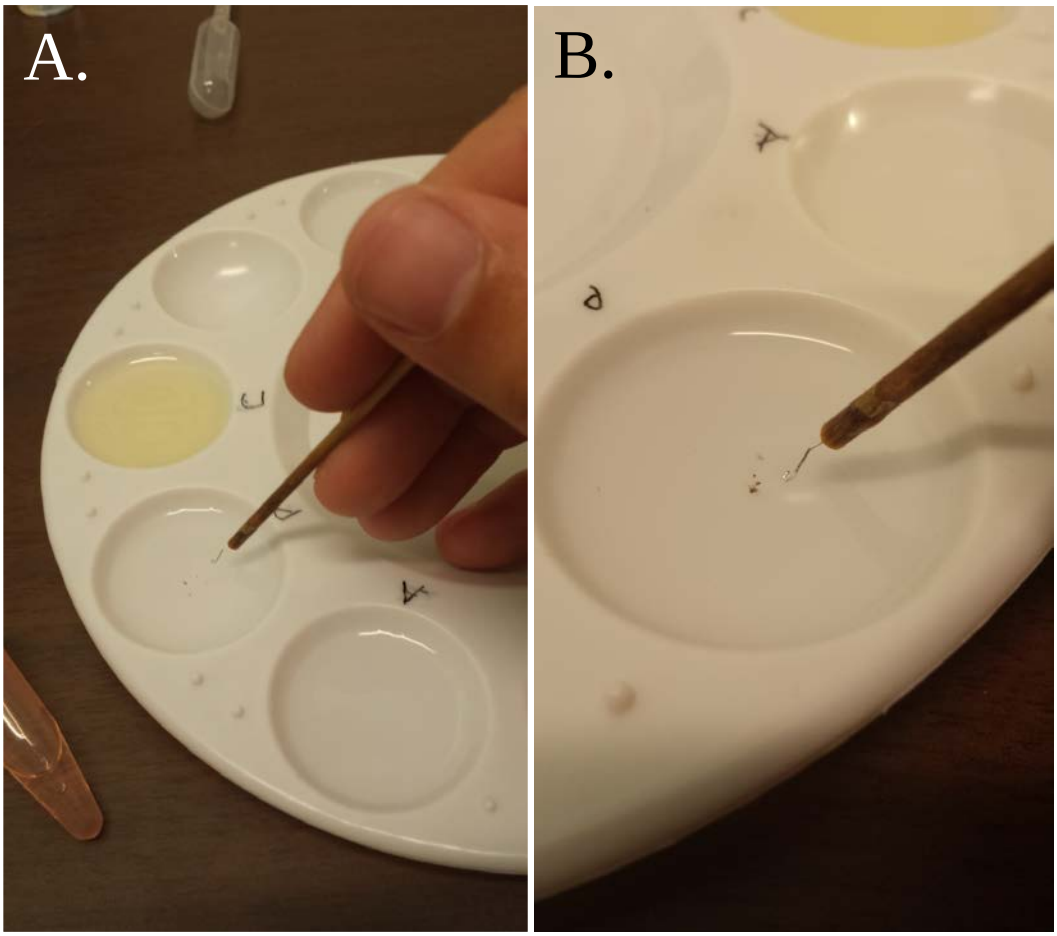

Fig. A-36. The process by which *Culicoides* are slide mounted. (A) Painters palette filled with acetic acid, “A”, propanol, “P”, and clove oil, “C”. (B) The tool used to transfer specimens to each well.

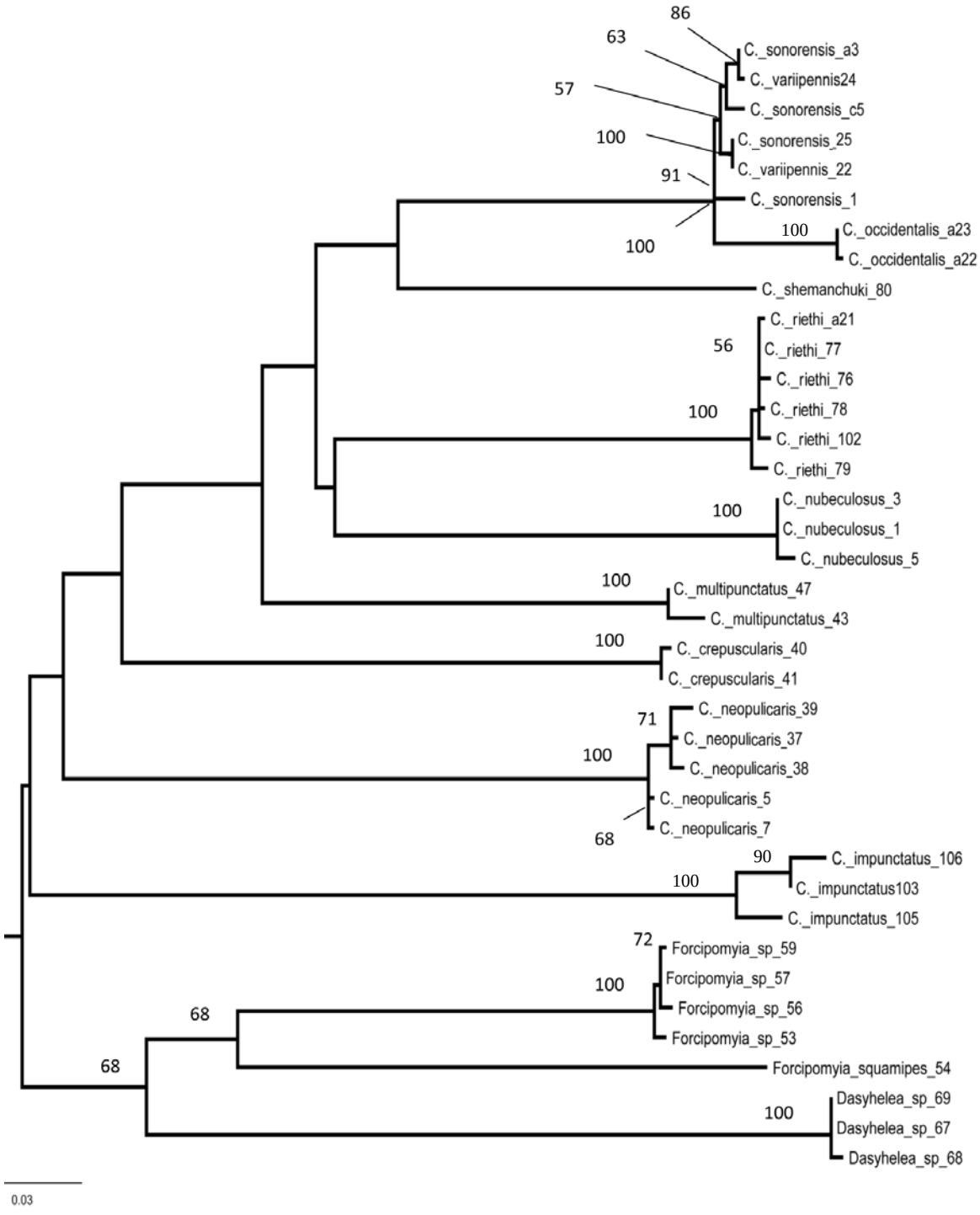
86
63
C._sonorensis_a3
C._variipennis24
57
C._sonorensis_c5
100
C._sonorensis_25
C._variipennis_22
91
C._sonorensis_1
100
100
C._occidentalis_a23
C._occidentalis_a22
C._shemanchuki_80
C._riethi_a21
56
C._riethi_77
C._riethi_76
100
C._riethi_78
C._riethi_102
C._riethi_79
100
C._nubeculosus_3
C._nubeculosus_1
C._nubeculosus_5
100
C._multipunctatus_47
C._multipunctatus_43
100
C._crepuscularis_40
C._crepuscularis_41
71
C._neopulicaris_39
C._neopulicaris_37
100
C._neopulicaris_38
C._neopulicaris_5
68
C._neopulicaris_7
90
C._impunctatus_106
100
C._impunctatus103
C._impunctatus_105
72
Forcipomyia_sp_59
100
Forcipomyia_sp_57
Forcipomyia_sp_56
68
Forcipomyia_sp_53
68
Forcipomyia_squamipes_54
100
Dasyhelea_sp_69
Dasyhelea_sp_67
Dasyhelea_sp_68
0.03

Fig. A-37. Cladogram of selected *Culicoides* species using COI sequences with bootstrap support, based of 2000 pseudoreplicates, and rooted with *Forcipomyia* and *Dasyhelea* outgroups (logL = -4163.06). Bootstrap vales of >50 are indicated for each clade.

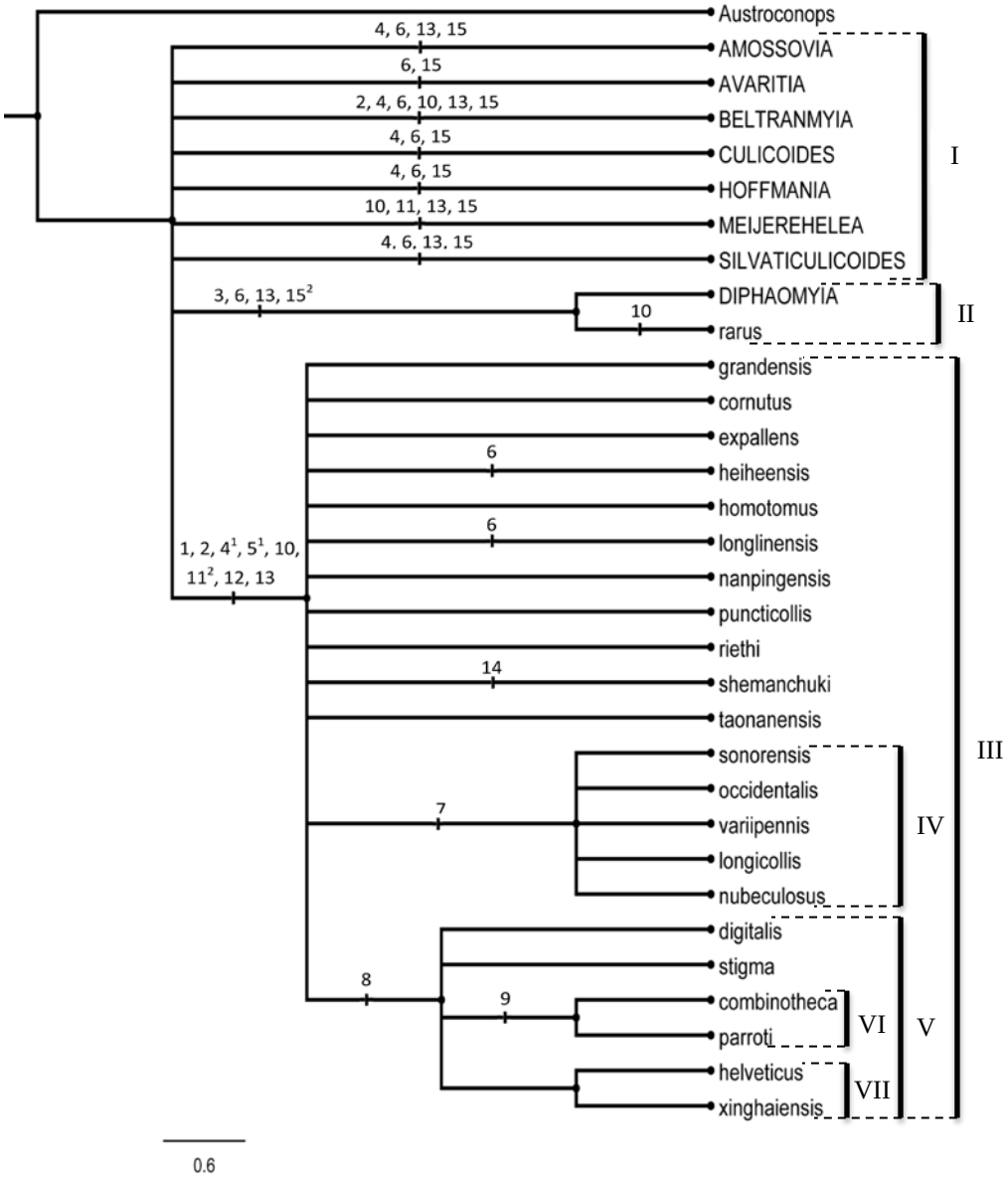
Austroconops
4, 6, 13, 15
AMOSSOVIA
6, 15
AVARITIA
2, 4, 6, 10, 13, 15
BELTRANMYIA
4, 6, 15
CULICOIDES
4, 6, 15
HOFFMANIA
10, 11, 13, 15
MEIJEREHELEA
4, 6, 13, 15
SILVATICULICOIDES
I
3, 6, 13, 15²
DIPHAOMYIA
10
rarus
II
grandensis
cornutus
expallens
6
heiheensis
homotomus
6
longlinensis
1, 2, 4¹, 5¹, 10,
11², 12, 13
nanpingensis
puncticollis
riethi
14
shemanchuki
taonanensis
III
sonorensis
occidentalis
7
variipennis
longicollis
nubeculosus
IV
digitalis
stigma
8
9
combinotheca
parroti
VI
V
helveticus
VII
xinghaiensis
0.6

Fig. A-38. A strict consensus tree of the 56 equally parsimonious trees found from unweighted parsimony analysis if data in Table B-1, rooted with an *Austroconops* outgroup, generated using TNT. The numbers above each branch represent a character state change. Superscripts were used to signify multiple state changes for a clade. For example, all members of Clade III share characters state 4, except for one species. Therefore it is written as 4^1. This method is used to show character state changes in Borkent (2014). Each clade within the tree is labeled, to the right, with a roman numeral.

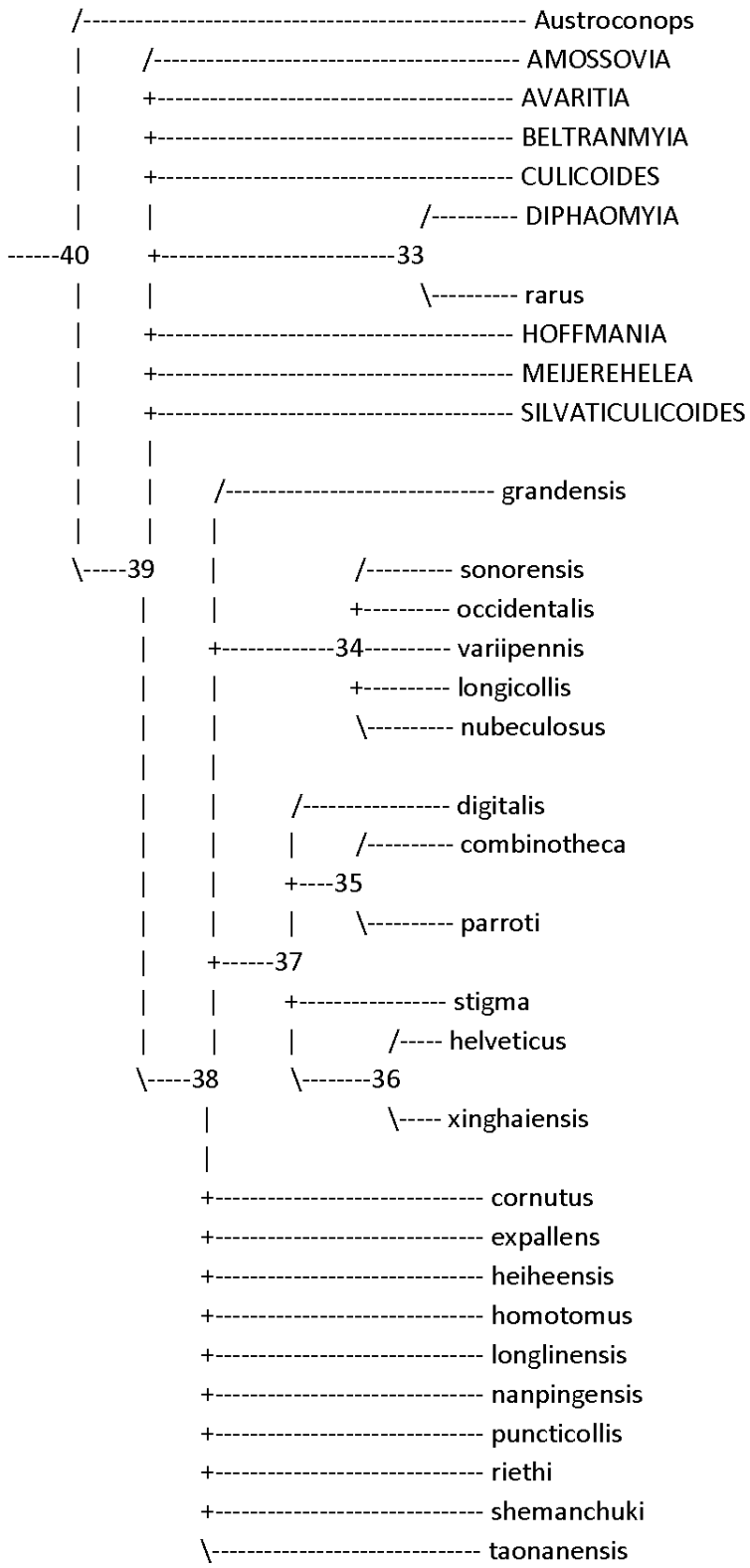
Austroconops
AMOSSOVIA
AVARITIA
BELTRANMYIA
CULICOIDES
DIPHAOMYIA
40
33
rarus
HOFFMANIA
MEIJEREHELEA
SILVATICULICOIDES
grandensis
39
sonorensis
occidentalis
34
variipennis
longicollis
nubeculosus
digitalis
combinotheca
35
parroti
37
stigma
helveticus
38
36
xinghaiensis
cornutus
expallens
heiheensis
homotomus
longlinensis
nanpingensis
puncticollis
riethi
shemanchuki
taonanensis

Fig. A-39. An unweighted strict consensus tree produced using the character matrix (Table B-1) rooted with an *Austroconops* outgroup generated by a parsimony analysis in PAUP*. The nodes (33 – 40) indicate an unambiguous state change. The total tree length is 34, consistency index is 0.4412, retention index is 0.7654, homoplasy index is 0.588, consistency index excluding uninformative characters is 0.4242, homoplasy index excluding uninformative characters is 0.5758, and rescaled consistency index is 0.3377.

APPENDIX B

Table B-1. Character matrix for *Culicoides* species

Taxon	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Austroconops	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AMOSSOVIA	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1
AVARITIA	?	0	0	0	0	1	0	0	0	0	0	0	0	0	1
BELTRANMYIA	?	1	0	1	0	1	0	0	0	1	0	0	1	0	1
CULICOIDES	?	0	0	1	0	1	0	0	0	0	0	0	0	0	1
***DIPHAOMYIA* sp. A**	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0
DIPHAOMYIA	?	0	1	0	0	1	0	0	0	0	0	0	1	0	1
HOFFMANIA	?	0	0	1	0	1	0	0	0	0	0	0	0	0	1
MEIJEREHELEA	?	0	0	0	0	1	0	0	0	1	1	0	1	0	1
SILVATICULICOIDES	?	0	0	1	0	1	0	0	0	0	0	0	1	0	1
combinotheca	?	1	?	?	?	?	0	1	1	1	1	?	1	0	0
cornutus	?	1	0	1	1	0	0	0	0	1	1	1	1	0	0
digitalis	?	?	0	1	1	0	0	1	0	1	1	?	1	0	0
expallens	?	?	0	1	1	0	0	0	0	1	1	?	?	0	0
grandensis	1	1	0	1	0	0	0	0	0	1	1	1	1	0	0
heiheensis	?	?	0	1	1	1	?	?	?	?	?	?	1	0	0
helveticus	?	1	0	1	1	0	0	1	0	1	0	?	1	0	0
homotomus	?	1	0	1	1	0	0	0	0	1	1	1	1	0	0
longicollis	?	1	0	1	1	0	1	0	0	1	1	1	1	0	0
longlinensis	?	1	0	0	1	1	0	0	0	1	1	1	1	0	0
nanpingensis	?	1	?	?	?	?	0	0	0	1	1	1	1	0	0
nubeculosus	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0
occidentalis	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0
parroti	?	1	0	1	1	0	0	1	1	1	1	1	1	0	0
puncticollis	?	1	0	1	1	0	0	0	0	1	1	1	1	0	0
rarus	?	0	1	0	0	1	0	0	0	1	0	0	?	0	0
riethi	1	1	0	1	1	0	0	0	0	1	1	1	1	0	0
shemanchuki	1	1	0	1	1	0	0	0	0	1	1	1	1	1	0
sonorensis	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0
stigma	?	1	0	1	1	0	0	1	0	1	1	1	1	0	0
taonanensis	?	1	0	1	1	0	0	0	0	1	1	?	1	0	0
variipennis	1	1	0	1	1	0	1	0	0	1	1	1	1	0	0
xinghaiensis	?	1	0	1	1	0	0	1	0	1	0	1	1	0	0

Table B-1. The character matrix used for phylogenetic analysis of *C.* (*Monoculicoides*). "0" denotes the absence of a character state, "1" denotes the presence of a character state, "?" denote an unknown character state.

Table B-2. List of Ceratopogonidae taxa

Genus	Subgenus	Species
Austropconops	-	*mcmillani*
Forcipomyia	*Forcipomyia*	*squamipes*
Forcipomyia	*unknown*	*unknown*
Dasyhelea	*unknown*	*unknown*
Culicoides	*Amossovia* sp. A	*arboricola*
Culicoides	*Amossovia* sp. B	*guttipennis*
Culicoides	*Amossovia* sp. C	*villosipennis*
Culicoides	*Avaritia* sp. A	*chiopterus*
Culicoides	*Avaritia* sp. B	*obsoletus*
Culicoides	*Avaritia* sp. C	*scotius*
Culicoides	*Beltranmyia* sp. A	*circumscriptus*
Culicoides	*Beltranmyia* sp. B	*hollensis*
Culicoides	*Beltranmyia* sp. C	*mississippiensis*
Culicoides	*Culicoides* sp. A	*impunctatus*
Culicoides	*Culicoides* sp. B	*newsteadi*
Culicoides	*Culicoides* sp. C	*punctatus*
Culicoides	*Diphaomyia* sp. A	*baueri*
Culicoides	*Diphaomyia* sp. B	*footei*
Culicoides	*Diphaomyia* sp. C	*haematopotus*
Culicoides	*Hoffmania* sp. A	*foxi*
Culicoides	*Hoffmania* sp. B	*insignis*
Culicoides	*Hoffmania* sp. C	*venustus*
Culicoides	*Meijerehelea* sp. A	*arakawai*
Culicoides	*Meijerehelea* sp. B	*guttifer*
Culicoides	*Silvaticulicoides* sp. A	*biguttatus*
Culicoides	*Silvaticulicoides* sp. B	*loisae*
Culicoides	*Silvaticulicoides* sp. C	*spinosus*
Culicoides	*Monoculicoides*	*combinotheca*
Culicoides	*Monoculicoides*	*cornutus*
Culicoides	*Monoculicoides*	*digitalis*
Culicoides	*Monoculicoides*	*expallens*
Culicoides	*Monoculicoides*	*grandensis*
Culicoides	*Monoculicoides*	*heiheensis*
Culicoides	*Monoculicoides*	*helveticus*
Culicoides	*Monoculicoides*	*homotomus*
Culicoides	*Monoculicoides*	*longicollis*
Culicoides	*Monoculicoides*	*longlinensis*
Culicoides	*Monoculicoides*	*nanpingensis*
Culicoides	*Monoculicoides*	*nubeculosus*
Culicoides	*Monoculicoides*	*occidentalis*

Table B-2 Continued

Culicoides	*Monoculicoides*	*parroti*
Culicoides	*Monoculicoides*	*puncticollis*
Culicoides	*Monoculicoides*	*rarus*
Culicoides	*Monoculicoides*	*riethi*
Culicoides	*Monoculicoides*	*shemanchuki*
Culicoides	*Monoculicoides*	*sonorensis*
Culicoides	*Monoculicoides*	*stigma*
Culicoides	*Monoculicoides*	*taonanensis*
Culicoides	*Monoculicoides*	*variipennis*
Culicoides	*Monoculicoides*	*xinghaiensis*

Table B-2. Species of Ceratopogonidae used in this study.

Table B-3. Apomorphy list

Branch			Character	CI	Change		
Node_40	→	Node_39	4	0.200	0	-->	1
			6	0.250	0	-->	1
			13	0.250	0	-->	1
			15	0.333	0	-->	1
Node_39	→	*Avaritia*	4	0.200	1	==>	0
			13	0.250	1	==>	0
Node_39	→	*Beltranmyia*	2	0.500	0	==>	1
			10	0.250	0	==>	1
Node_39	→	*Culicoides*	13	0.250	1	==>	0
Node_39	→	*Hoffmania*	13	0.250	1	==>	0
Node_39	→	*Meijerehlea*	4	0.200	1	==>	0
			10	0.250	0	==>	1
			11	0.333	0	==>	1
Node_39	→	Node_33	3	1.000	0	==>	1
			4	0.200	1	==>	0
Node_33	→	*rarus*	10	0.250	0	==>	1
			15	0.333	1	==>	0
Node_39	→	Node_38	1	1.000	0	==>	1
			2	0.500	0	==>	1
			5	0.500	0	==>	1
			6	0.250	1	==>	0
			10	0.250	0	==>	1
			11	0.333	0	==>	1
			12	1.000	0	==>	1
			15	0.333	1	==>	0

Table B-3 Continued.

Branch			Character	CI	Change		
Node_38	→	*grandensis*	5	0.500	1	==>	0
Node_38	→	*heiheensis*	6	0.250	0	==>	1
Node_38	→	*longlinensis*	4	0.200	1	==>	0
			6	0.250	0	==>	1
Node_38	→	*shemanchuki*	14	1.000	0	==>	1
Node_38	→	Node_34	7	1.000	0	==>	1
Node_38	→	Node_37	8	1.000	0	==>	1
Node_37	→	Node_35	9	1.000	0	==>	1
Node_37	→	Node_36	11	0.333	1	==>	0

Table B-3. Apomorphy list generated in PAUP*. Each branch from Fig. A-39 is listed with the corresponding character or characters, CI value of each character, and the state changes (0, 1). Unambiguous character state changes are denoted with a double lined arrow (==>).

Table B-4. List of species used in genetic analysis

Taxon		Location		Collection Date	Ascension number
Culicoides	*sonorensis*	Texas	Grimes Co.	10.VII.14	KT794137
Culicoides	*sonorensis*	Texas	Grimes Co.	10.VI.14	KT794141
Culicoides	*sonorensis*	Kansas	"AK" colony	25.I.14	KT794144
Culicoides	*sonorensis*	Texas	Grimes Co.	10.VII.14	KT794159
Culicoides	*variipennis*	Texas	Grimes Co.	16.VII.14	KT794138
Culicoides	*variipennis*	Texas	Grimes Co.	10.VII.14	KT794161
Culicoides	*occidentalis*	Canada	BC	4.V.14	KT794140
Culicoides	*occidentalis*	Canada	BC	4.V.14	KT794158
Culicoides	*shemanchuki*	Canada	AB	4.IX.13	KT794134
Culicoides	*riethi*	Canada	AB	4.IX.13	KT794139
Culicoides	*riethi*	Canada	AB	4.IX.13	KT794153
Culicoides	*riethi*	Canada	BC	4.V.14	KT794157
Culicoides	*riethi*	Canada	AB	4.IX.13	KT794160
Culicoides	*riethi*	Canada	BC	4.V.14	KT794165
Culicoides	*riethi*	Canada	AB	4.IX.13	KT794166
Culicoides	*nubeculosus*	England	Lab colony	1.IV.14	KT794135
Culicoides	*nubeculosus*	England	Lab colony	1.IV.14	KT794136
Culicoides	*nubeculosus*	England	Lab colony	1.IV.14	KT794163
Culicoides	*multipunctatus*	Texas	Burleson Co.	14.VIII.14	KT794154
Culicoides	*multipunctatus*	Texas	Grimes Co.	22.V.14	KT794155
Culicoides	*crepuscularis*	Texas	Grimes Co.	18.III.14	KT794142
Culicoides	*crepuscularis*	Texas	Grimes Co.	18.III.14	KT794143
Culicoides	*neopulicaris*	Texas	Grimes Co.	16.VI.14	KT794162
Culicoides	*neopulicaris*	Texas	Grimes Co.	10.VII.14	KT794164
Culicoides	*neopulicaris*	Texas	Grimes Co.	10.VII.14	KT794165
Culicoides	*neopulicaris*	Texas	Grimes Co.	5.VI.14	KT794167
Culicoides	*neopulicaris*	Texas	Grimes Co.	5.VI.14	KT794171
Forcipomyia	*sp.*	Texas	Burleson Co.	3.VII.14	KT794148
Forcipomyia	*sp.*	Texas	Burleson Co.	3.VII.14	KT794150
Forcipomyia	*sp.*	Texas	Burleson Co.	3.VII.14	KT794151
Forcipomyia	*sp.*	Texas	Grimes Co.	5.VI.14	KT794152
Forcipomyia	*squamipes*	Texas	Grimes Co.	12.VI.14	KT794149
Dasyhelea	*sp.*	Texas	Grimes Co.	30.VII.14	KT794168
Dasyhelea	*sp.*	Texas	Grimes Co.	30.VII.14	KT794169
Dasyhelea	*sp.*	Texas	Grimes Co.	30.VII.14	KT794170
Culicoides	*impunctatus*	Ireland	Kerry	1.VI.13	KT794145
Culicoides	*impunctatus*	Ireland	Kerry	1.VI.13	KT794146
Culicoides	*impunctatus*	Ireland	Kerry	1.VI.13	KT794147

Table B-4. List of species used in genetic analysis, with locality, collections date, and GenBank ascension number.