The female genitalia of Tipulidae, Trichoceridae, Ptychopteridae and Mycetophilidae (Diptera)

Master of Science Thesis in Ecology and Evolution

Trude Magnussen

The Natural History Museum

University of Oslo

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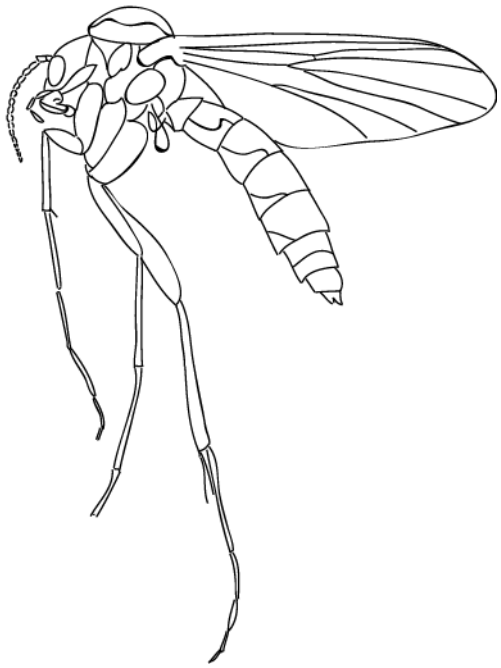

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Trude Magnussen

31th May 2013, Oslo

Contents

Preface

The main objective of the present study is to describe and compare the morphology of the female genitalia within a selected number of species and families of the lower Diptera. The results are presented as two papers with a common introduction. The first paper investigates the differences at the family level, based on four species representing the three families Tipulidae, Trichoceridae and Ptychopteridae. The second paper focuses on differences between species within one genus in the family Mycetophilidae. Females of four species in the genus *Allodia* Winnertz, 1863 were selected for this part of the study. The females are associated with already identified males through DNA barcoding. The common introduction gives a short review of the phylogeny of the studied taxa, the outline of female genitalia in lower Diptera and of DNA barcoding as a method for associating sexes.

The achieved results suggest that a more thorough study of female genitalia both at species-level and higher taxonomical levels is likely to reveal taxonomical important characters.

Introduction

Within entomology, the study of insect genitalia has always been an important tool in classification and in identification of species, families, groups and genera. The male genitalia seem to be rapidly changing in an evolutionary perspective compared to other structures of the body and are thus excellent for differentiating between species (Eberhard 1985; Grimaldi and Engel 2005). It is assumed that sexual selection is the most important reason for this tremendous plasticity in the morphology of the male genitalia (Arnqvist 1998). In Diptera, as in several other insect orders, the male genitalia hold many of the most important characters for species-level taxonomy. However, because of the large variation in the male genital structures in Diptera, it can be extremely difficult to designate homologous structures between taxa (van Emden and Hennig 1970). Differences in the female genitalia on the other hand, are less pronounced, especially on the species level (Scudder 1971; Eberhard 1985). The subtle differences found in the genital structures of females in several insect taxa are probably due to weaker selection pressure (Eberhard 1985; 2010). The female genitalia of Diptera have generally been presumed to be of little systematic importance at the species-level, which partly explain why the genitalia have been less studied in females than in males. As the female genitalia are believed to change more slowly than the male genitalia, genital characters in females may expose traits that may be more suitable for comparisons at a higher taxonomical level, and thus give a unique possibility to recognize homologies, as done by Sæther (1977).

Phylogeny

Diptera was until recently divided in the two suborders, Nematocera and Brachycera, and this division is still in use in more common presentations of the order. This distinction was largely based upon differences in adult antennae. Nematocera are now considered paraphyletic with respect to Brachycera. In this study the group will be referred to as the lower Diptera. The phylogenetic relationships between the families in this group have been a topic of debate and controversy, and consensus about the interfamiliar relationship is still not established. New methods, in particular molecular data and a broader taxon sampling have brought us closer to a better understanding and a fully resolved tree (Wiegmann *et al.* 2011). According to traditional classification, the lower Diptera consists of five suborders (infraorders); Ptychopteromorpha, containing the single family Ptychopteridae; Tipulomorpha, containing

Tipuloidea and Trichoceridae; Psychodomorpha; Culicomorpha; and Bibionomorpha, where among others, Mycetophilidae is found (Yeates and Wiegmann 1999). In addition to the suborders mentioned, there are four enigmatic families; Deuterophlebiidae, Nymphomyiidae, Axymyiidae and Perissommatidae, with a very uncertain phylogeny. In the discussion concerning the phylogeny of Diptera, only a selected number of hypotheses will be discussed, i.e. Wiegmann *et al.* (2011), Petersen *et al.* (2010), Bertone *et al.* (2008), Bertone (2008), Yeates and Wiegmann (2005), Oosterbroek and Courtney (1995) and Wood and Borkent (1989). Both phylogenies based on morphology and molecular analyses will be considered, with a focus on the groups included in this study. A more complete review can be found in Yeates and Wiegmann (2005).

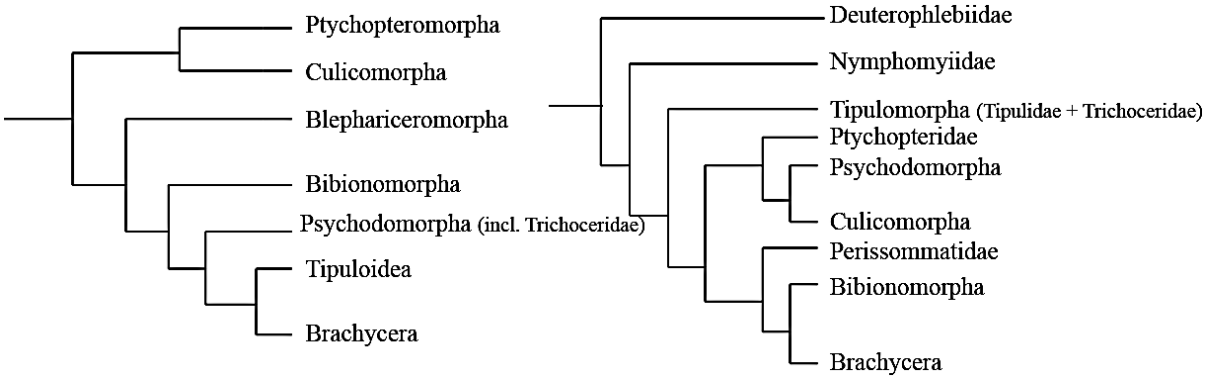

Figure 1: LEFT: Phylogeny of lower Diptera, modified from Yeates and Wiegmann (2005). **RIGHT**: Phylogeny of the Lower Diptera, modified from Wiegmann *et al*. (2011)

The families Pediciidae, Cylindrotomidae, Tipulidae (=Tipulidae *sensu stricto*) and Limoniidae have traditionally been included in the superfamily Tipuloidea (Petersen *et al.* 2010). Molecular data, however, does not support a monophyletic Limoniidae, hence the traditional four family system is rejected (Bertone 2008; Petersen *et al.* 2010). In the phylogenetic analysis by Wood and Borkent (1989) Tipuloidea, or Tipulidae *sensu lato*, is considered to be the only member of Tipulomorpha, and was positioned at the basis of the Diptera. Tipuloidea has also been treated as more derived, as a sistergroup to Brachycera (see Fig. 1) (Yeates and Wiegmann 2005).

The placement of Trichoceridae has varied considerably over the last fifty years. Two alternative positions of Trichoceridae have been suggested. Traditionally the family has been placed in the suborder Tipulomorpha, together with Tipuloidea, which is in accordance with recent studies (Bertone *et al.* 2008; Wiegmann *et al.* 2011) (Fig. 1). However, the alternative position is together with Anisopodidae in Psychodomorpha, as in the studies of Wood and Borkent (1989) and Yeates and Wiegmann (2005) (Fig. 1). The latter position is also in accordance with older studies based on morphological characters, see e.g. Crampton (1926).

The placement of Tipulomorpha (including Tipuloidea and Trichoceridae) within Diptera is also uncertain. This is illustrated in the study of Bertone *et al.* (2008) which, as mentioned, find good support for the suborder Tipulomorpha, but the position of the suborder within the Dipteran phylogeny could not be resolved. According to Wiegmann *et al.* (2011), Deuterophlebiidae is the most basal group of Diptera. Nymphomyiidae is the sister of the six traditional suborders, and Tipulomorpha is placed at the base (see Fig. 1). The basal placement of Tipulomorpha is in accordance with one of the earliest phylogenetic hypotheses of the suborder, proposed by Hennig (1973).

Based on morphology, Ptychopteridae was included in the infraorder Ptychopteromorha, together with Tanyderidae (Wood and Borkent 1989). A sister group relationship with Tanyderidae has also been proposed by several authors, including Oosterbroek and Courtney (1995). What is recognized as the most plausible situation today is that Ptychopteridae is the sole family in the Ptychopteromorpha, while Tanyderidae is placed in the Psychodomorpha together with Blephariceridae and Psychodidae. This result is supported by molecular studies and combined morphological and molecular analyses (Bertone *et al.* 2008; Wiegmann *et al.* 2011) (see Fig. 1). There is still controversy concerning the position of Ptychopteromorpha with respect to other families and infraorders. Bertone *et al.* (2008) propose Ptychopteridae to be an early-diverging and independent lineage of flies. This is also the outcome in the combined molecular phylogenetic supertree in Wiegmann *et al.* (2011), where Ptychopteridae is regarded as the sister group to Psychodomorpha and Culicomorpha, but as a separate suborder.

Mycetophilidae belongs to the suborder Bibionomorpha, The number of includes families in Bibionomorpha has varied through time, and there is still no full consensus on this subject. Based on morphological characters, Wood and Borkent (1989) included the three superfamilies Pachyneuroidea (with one family, Pachyneuridae), Bibionoidea (with one

family, Bibionidae) and Sciaroidea (with 3 families, Mycetophilidae, Sciaridae and Cecidomyiidae). This is manly in accordance with the molecular findings presented by Wiegmann *et al.* (2011). Later Axymyidae was included in Bibionmorpha by Oosterbroek and Courtney (1995). The family Mycetophilidae has subsequently been divided into several smaller families, varying from 7 to 9 between authors. The relationships between the families in Bibionomorpha are uncertain, but the infraorder is regarded as a derived group within the lower Diptera and in the combined molecular phylogenetic tree of Diptera in Wiegmann *et al.* (2011), Bibionomorpha forms the sister group to Brachycera and includes the families; Anisopodidae, Canthyloscelidae, Scatopsidae, Axymyiidae, Bibionidae, Pachyneuridae, Ditomyiidae, Manotidae, Diadocidiidae, Sciaridae, Cecidomyiidae, Lygistorrhinidae, Mycetophilidae, Keroplatidae and Bolitophilidae (see Fig. 1).

Outline of the female genitalia in lower Diptera

The abdomen of Diptera is primitively composed of 11 segments (McAlpine 1981), as for insects in general. The number of segments is commonly reduced in higher Diptera, but such reduction has also been found in the lower Diptera (Matsuda 1976). The abdomen can be partitioned in to three parts: the pre-genital segments, the genital segments and the post-genital segments. The genital and post-genital segments are often referred to as the terminalia, which is defined as the modified genitalia and any adjacent segment that show modification for copulation or oviposition (McAlpine 1981). The genital opening in female Diptera is primitively located between segment 8 and 9, and these segments are therefore referred to as the genital segments. Segments 1 to 7 are the pre-genital segments, each with a pair of spiracles laterally and a generally rather unmodified and homogenous outline. The post-genital segments are segments or parts posterior to segment 9, commonly referred to as the proctiger. In this study, however, proctiger is interpreted in the narrow sense, i.e. as consisting only of the parts behind segment 10. Segment 10 and the proctiger will thus be treated separately, following e.g. Sæther (1977) and Søli (1997). The proctiger consists of the epiproct (the tergal part of segment 11); the hypoproct (the sternal part of segment 11); and the cerci. The cerci are defined as a paired appendage placed dorsally, on both sides of the anus, they are considered derived from the proctiger (Cumming and Wood 2009). In most female lower Diptera the cerci are two-segmented, but in some families they are one-segmented. Two-segmented cerci are considered the most primitive state in both the lower

Diptera and Brachycera (McAlpine 1981). The hypoproct is commonly fused with vestiges of sternite 10, located at the ventral base of the cerci.

Segments 8 and 9 are highly specialized in females of the lower Diptera. They each consist of a tergal and sternal part in addition to a pair of gonopods (Crampton 1929; Smith 1969; Sæther 1977). In the female, the gonopods are composed of a basal pair of gonocoxites (valvifers) and a pair of gonapophyses (valvulae). The tergal parts of the terminalia are generally less modified than the sternal parts. Each segment is often easily recognized, although reductions and fusions occur. The outline of tergite 8 is often similar to the pre-genital segments, while tergite 9 is usually more modified, and is occasionally reduced. Sternite 8 bears a pair of gonocoxites caudally, but often it is not possible to separate the gonocoxite from the sternite. Principally, the gonocoxites are found at the basis of gonapophyses 8. The gonapophyses 8 are often reduced or fused in lower Diptera, but still recognizable. The gonocoxites of segment 9 are more conspicuous but commonly associated with tergite 9 and are partly fused along the lateroventral part of the tergite. Gonapophyses 9 and the remnants of sternite 9 are located at the dorsal wall of the genital chamber. This composite structure surrounds the opening of the spermathecae. Its shape varies, but it frequently contains both sclerotized and membranous parts. The inner, lateral border of each of the two gonapophyses 9 is usually more strongly sclerotized, and commonly fused distally, forming a more or less prominent notum (as described in Sæther 1977). The spermathecae are sperm storage organs. The primitive number is three, but the number varies from one to four in some groups (Downes 1968). The spermathecae are derived from segment 8, and open near the primary gonopore, close to sternite and gonapophyses of segment 9 (Cumming and Wood 2009).

DNA barcoding as a tool for species identification

DNA barcoding of animals was suggested by Hebert *et al.* (2003) as a compensation for the shortage of taxonomic expertise ("the taxonomic impediment"). Using standardized DNA regions as barcodes can be a powerful, quick and cost-effective tool for species identification provided that well-developed DNA barcode libraries exist. DNA barcoding has two main objectives, firstly to identify individuals using molecular methods based on a reference library of known species, and secondly to examine unknown biodiversity, with the aim to describe new species (Hebert *et al.* 2003). The molecular marker suggested by Hebert *et al.* (2003) was

the mitochondrial, protein coding gene; cytochrome c oxidase subunit 1 (COI), which today is used as the standard barcode region for animal taxa. COI evolves quickly enough to separate closely related species (Blaxter 2003) and it has an advantage over the commonly used ribosomal genes 12S and 16S because it lacks indels (insertions and deletions) (Hebert *et al.* 2003). Furthermore robust primers have been designed for COI, which work on a broad range of metazoan invertebrates (Folmer *et al.* 1994). As the method has been tested and used for about a decade, some challenges have been encountered, which illustrate that divergence in COI is not necessarily synonymous with different species (Hogner *et al.* 2012; Kvie *et al.* 2013). Incomplete lineage sorting causes the gene tree, the genealogy, to differ from the species tree, the phylogeny. A solution to this is to compare several genes and subsequently get a more trustworthy result, where the gene tree is in accordance with the species tree (Egan and Crandall 2006; Alexander *et al.* 2009). Divergence in COI may also be due to nuclear pseudogenes of mitochondrial origin (so-called numts – nuclear mitochondrial DNA). A pseudogene is a sequence which is similar to a normal gene, but not functional, therefore it can contain mutations that inhibit the production of proteins (Fox and Wolf 2006). A solution to the challenge of pseudogenes is to check for double peaks, frameshift mutations and stop codons (Song *et al.* 2008).

By using DNA barcoding it is possible to identify and associate gender and immature stages of a species (Blaxter 2004). A common problem when studying Diptera is that frequently only one sex is known and described, usually the male. DNA barcoding has shown to be a very important tool in biodiversity estimates e.g. in Ekrem *et al.* (2010) and Stur and Ekrem (2011), where it is important to identify both sexes and several life stages in Chironomidae. Other commonly used methods for associating sexes, such as morphology, locality of sampling and hatching can be time consuming and requires specialized taxonomic skills. Associating sexes based on morphology can be difficult due to sexual dimorphism and hatching can be extremely time consuming and challenging depending on the group of study, as laboratory conditions may not suit the requirement of the species. The COI gene fragment has been successfully used in several phylogenetic analyses of the Mycetophilidae (Rindal *et al.* 2007; 2009), it has also proven to be successful in association of sexes within the family (Kurina *et al.* 2011).

Objectives

In this study, both variation between families and between closely related species are investigated. Three families of the lower Diptera, Tipulidae, Trichoceridae and Ptychopteridae, have been chosen for the purpose of comparing the female genitalia at the family-level. The prediction is that the homologous structures found in the female genitalia are more easily observed and recognized, due to their pleisomorphic outline. Association of sexes through DNA barcoding is demonstrated in this thesis for the genus *Allodia* (Mycetophilidae) and the interspecific variation in female genitalia between four species from the genus is examined. The prediction is that there will be differences at the species level, although more modest than what is found at the family-level.

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Manuscript 1: Comparative morphology of the female genitalia in Tipulidae, Trichoceridae and Ptychopteridae (Diptera)

Abstract

The female genitalia of four species of lower dipterans, *Ptychoptera minuta* Tonnoir, 1919, *Trichocera major* Edwards, 1921, *Tipula scripta* Meigen, 1830 and *Tipula variicornis* Schummel, 1833, from the three families Ptychopteridae, Trichoceridae and Tipulidae are described, illustrated and compared. A common terminology is applied to the structures reflecting the homology of the observed parts. By comparing the female genitalia of the three families, homologous structures as well as structures unique to each family are pointed out and discussed. It was found that the genital structures generally expose several modifications, fusions, reductions and shift in positions. The internal structures (derived from sternite 9) were found to be the most polymorphous, in addition to reductions and fusions of the tergal structures. The most divergent outline was found in Ptychopteridae, where the tergal sclerites are clearly reduced, but with sternite 8 and gonocoxites 8 well-developed. Furthermore, the internal genital structures in Ptychopteridae are highly complex and difficult to interpret and homologize. Tipulidae and Trichoceridae are more similar in their outline, and they both have well-developed gonapophyses 8 and sternite 10, which appear to be lost in Ptychopteridae. The fusion of tergite 8 and tergite 9 is unique to Trichoceridae compared to the two other families. Clear and well developed gonocoxites 9 and hypoproct could only be found in Tipulidae. These results suggest that structures of both systematical and taxonomical importance can be retrieved through a more thorough examination of female genitalia in the lower Diptera.

Introduction

The insect genitalia form a highly composite structure that varies considerably between taxonomical groups, not only between orders and families, but even between species within one genus (Eberhard 1985). In Diptera, like in many other insect orders, the male genitalia enables researchers to distinguish between species that otherwise have almost identical external morphology. This is clearly illustrated in the study of Grimaldi and Nguyen (1999). The female genitalia in Diptera on the other hand, are largely understudied, which is also reflected in the vague terminology used to describe these structures, where different authors to a large extent use different terminologies depending on the group in question (Sæther 1977). The reason why female genitalia are less studied is that researchers generally consider female genitalia to be relatively uniform and of minor interest when separating between species (Eberhard 1985). On the other hand, a slow differentiation of parts and structures can give good possibilities to recognize homologies at a higher taxonomic level.

In the present study, the female genitalia are described and compared at the family-level. The female genitalia of four species from the three families Tipulidae, Trichoceridae and Ptychopteridae are described, illustrated and compared. The hypothesis is that a comparative study of the female genitalia can provide new insights to the evolution of the parts, their homology, as well as structures unique to each family.

The results are compared with previous studies of the female genitalia within the lower Diptera, with special reference to studies dealing with the families being at target in this study, e.g. Rees and Ferris (1939); Peus (1958); Byers (1961); Frommer (1963); Dahl (1980); McAlpine (1981); Andersson (1997), in addition to descriptions dealing with the family Mycetophilidae (Väisänen 1984; Blaschke-Berthold 1994; Søli 1997). The descriptions of female genitalia in the family Mycetophilidae in Søli (1997) serves as a foundation for the present interpretation of the female genital structures. Different terminologies are used to describe the female genital structures in different families and these terminologies do frequently coincide. To enable an unambiguous comparison of the outline of the female genitalia in the three families, attempts are made to apply a common terminology, reflecting the homology between the observed structures. The issue about the origin of the genital structures, however, falls outside the scope of this study, and is only briefly discussed to highlight the major hypotheses.

Origin and homology of the female genital structures in Diptera

Snodgrass (1935) describes the basic structures of the female external genitalia of Pterygota as being composed of the first and second valvulae; present at the base of these valvulae are the first and second valvifers (see Fig. 1). The first valvifers are associated with segment 8, and the second valvifer with segment 9. The valvifers are considered to be homologue to the gonocoxites, and the valvulae to the gonapophyses (Snodgrass 1935) (see Fig. 1). The terms gonapophysis and gonocoxite are used in this thesis. The gonapophyses probably represent unique innovations within Insecta, while the gonocoxites can be homologized with limb appendages (Matsuda 1976). In Hemimetabola the gonapophyses form an ovipositor and Hymenoptera is the only holometabolous order which has retained an ovipositor of gonapophyseal origin (Mickoleit 1973; Hünefeld *et al.* 2012). The term ovipositor literary means a device for egg-laying and in that sense an ovipositor is also present in Diptera. Hünefeld *et al.* (2012) considers the same elements present in the ovipositor of Hymenoptera to also be present in non-hymenopteran orders of Holometabola, but refer to them as genital appendages. In many families of the lower Diptera a functional ovipositor is made up of the cerci and sternite 8/gonocoxite 8, together with gonapophyses 8 and 9.

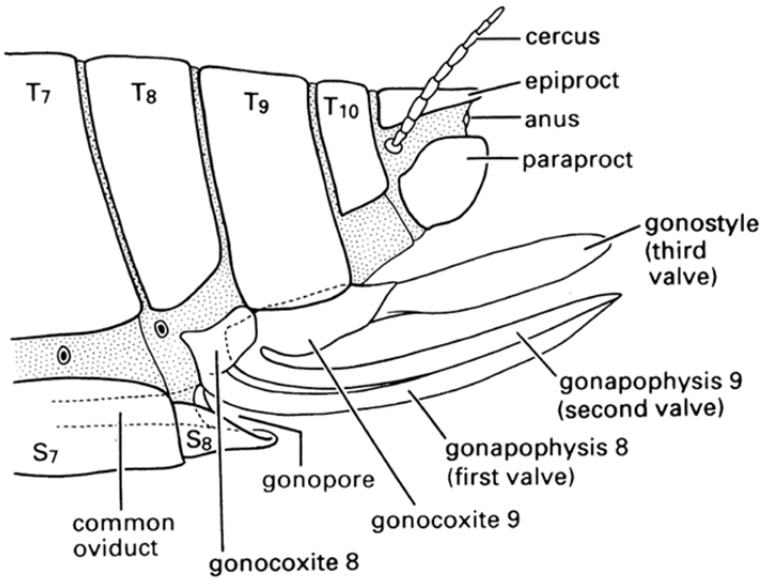

Figure 1: Generalized ovipositor of Orthoptera, lateral view. Figure from Gullan and Crantson (2010), after Snodgrass (1935). Showing appendages (gonocoxites and gonapophyses) of segments 8 and 9, valve =valvulae. **Abbreviations:** S = sternite; T = tergite

Evolution of structures and organs involves differentiation, production of new structures, shift in position, fusion, reduction and loss (Matsuda 1976). All of these processes are directed by natural selection, in which sexual selection plays an important role in the evolution of

genitalia (Eberhard 1985; Arnqvist 1998). According to definition, a structure is homologous if it occurs in two or more species (descendants) and is derived from a common ancestor, with or without modification (Grimaldi and Engel 2005). Several authors consider the gonapophyses and gonocoxites to have evolved independently within Diptera and thus should not be regarded homologous to the structures observed in Hymenoptera, see e.g. Crampton (1929) and Frommer (1963). It is uncertain whether or not the gonapophyses and gonocoxites in Diptera can be considered homologous to the structures found in the ovipositor of Hemimetabola and Hymenoptera. Even so, it is reasonable to believe that since the gonapophyses and gonocoxites are well developed structures in the ground plan of Holometabola, these structures might also be present in the ground plan of Diptera, although modified, reduced or fused with other structures. This interpretation is in accordance with Hünefeld *et al.* (2012). By using the term gonapophyses this will also imply that gonocoxites are, or have been present. The term gonocoxite is not frequently used in females, but it has been used by both Sæther (1977) and several authors dealing with Mycetophilidae, e.g. Søli (1997).

Ptychopteridae

Ptychoperidae is a small family of Diptera, with approximately 70 species described worldwide (Wagner *et al.* 2008). Despite being few in number they are distributed in most biotic regions, except the Australian (Alexander 1981a); and they were not recorded from the Neotropical region until 2006 (Hancock *et al.* 2006). About 13 species are present in Europe (Andersson 1997). The adults resemble tipulids, but with a somewhat stouter, darker and more lustrous body. The presence of the prehaltere is their most characteristic feature. They have one spur on the fore tibia and two distinct tibial spurs on the middle and hind leg, no ocelli and long antennae with 16 segments (Alexander 1981a). The larvae develops in detritus in lakes, ponds and streams and is restricted to shallow water because of the length of respiratory tube (Alexander 1981a). The adults are usually found near the breeding places, and are quite sedentary, often resting on vegetation during the day, but can be more active during early morning and evening (Andersson 1997). Little is known about their feeding habits as adult, but they probably feed on honeydew and nectar (Shcherbakov and Lukashevich 2005).

Ptychopteridae are split in two subfamilies; Ptychopterinae and Bittacomorphinae (Lukashevich 2012). Ptychopterinae are monogeneric, only containing the genus *Ptychoptera*

Meigen, 1803. Bittacomorphinae contains two genera; *Bittacomorphella* Alexander, 1916 and *Bittacomorpha* Westwood, 1835.

There are few detailed descriptions of the female genitalia, but detailed drawings and descriptions are present in the work by Rozkosny (1997), which is largely based on the study by Peus (1958). Andersson (1997) gives detailed illustrations of the female genitalia in all species of North European Ptychopteridae. The terminology used by all authors is descriptive, with no intention to assess origin or homology of the observed structures.

Trichoceridae

Trichoceridae is recorded from all biotic regions, but no species have yet been recorded from the African continent (Dahl and Alexander 1976). The fauna is best documented in the West Palearctic, East Nearctic and Australia, with about 157 species described worldwide (Dahl and Krzeminska 1997). Trichoceridae are small to medium sized flies (average body size from 3 to 8 mm), with long abdomen and long, slender legs (Dahl and Krzeminska 1997). The antennae are long, with 16 flagellomeres, and there are three ocelli present (Alexander 1981b). The larvae are terrestrial and feed on decaying plant material, rotten wood and fungi (Alexander 1981b). In the northern hemisphere the genus *Trichocera*, are commonly called winter crane flies due to their swarming during fall, winter and spring months (Dahl and Krzeminska 1997).

Trichoceridae are divided into two subfamilies; Trichocerinae and Paracladurinae (Krzeminska 2009). Trichocerinae includes *Trichocera* Meigen, 1803, *Diazosma* Bergroth, 1913 and *Nothotrichocera* Alexander, 1926, while Paracladurinae includes the single genus *Praracladura* Brunetti, 1911. The genus *Trichocera* is further divided into three subgenera; *Trichocera* Meigen 1803, *Metatrichocera* Dahl, 1866 and *Saltrichocera* Krzeminska, 2002.

The females have been described for several species of Trichoceridae, and for some the species description is even based on females solely. Dahl (1980) conducted a study on the postembryonic organization of the genital segments in Trichoceridae, Tipulidae and Anisopodidae, by studying sections of different larval instars and the adult. The terminology of Dahl is followed by numerous authors in their descriptions of the female genitalia within the family. McAlpine (1981) suggests an alternative terminology for the genital parts in

Trichoceridae, and even use Trichoceridae as an example of the outline found in the lower Diptera.

Tipulidae

Tipulidae *sensu stricto* is a large and diverse family of flies, with more than 4000 described species worldwide (de Jong *et al.* 2008). They are adapted to many different habitats. Most species are associated with freshwater, but species can also found in forests, intertidal zones and mountain areas. Tipulidae are large to medium sized flies, with long abdomen and legs. They can be recognized on their wing venation, a pronounced v-shaped, transverse mesonotal suture, four segmented palpus with the last segment elongated, antennae with 13 flagellomeres and no ocelli present (Alexander and Byers 1981). Their larval stages are often linked to freshwater or moist habitats; but some species can also be found in dry areas (Alexander and Byers 1981).

The phylogeny of Tipulidae has been extensively discussed. Originally the superfamily Tipuloidea included the four families Tipulidae *sensu stricto*, Pediciidae, Cylindrotomidae and Limoniidae. This four family system has been questioned by several authors, e.g. Bertone (2008), and larger studies including many characters from both morphology and molecular analyses have rejected the hypothesis (Bertone *et al.* 2008; Petersen *et al.* 2010). Several of the problems associated with the phylogeny are due to the paraphyly or polyphyly of Limoniidae. Petersen *et al.* (2010) proposes a superfamily Tipuloidea, containing two families; Pediciidae and Tipulidae (including Tipulidae *sensu stricto*, Cylindrotomidae and Limoniidae, all treated as subfamilies). In this study however Tipulidae *sensu stricto* is treated as a separate family, in accordance with the most common interpretation.

Several authors have described the female genitalia in Tipulidae and many hypotheses concerning the homology of the female genitalia have been proposed (Snodgrass 1903; Rees and Ferris 1939; Byers 1961; Frommer 1963; de Jong 1997). The female genitalia of Tipulidae is commonly used in comparative studies as a model of lower Diptera, see e.g Dahl (1980) and Hünefeld *et al.* (2012). This is due to the presumably basal position of this group in the dipteran phylogeny.

Material and Methods

Material

The species chosen are supposed to reflect the general outline within each family. In Trichoceridae and Ptychopteridae one species of each was chosen, as all studied species displayed a very similar outline. For Tipulidae two species were chosen to illustrate two rather different outlines found in the available material. All studied material was preserved in 80 % ethanol, and the specimens were stored together with the microscope slides, or micro vials containing the terminalia, at the Natural History Museum, University of Oslo. The material includes all studied species in addition to the ones illustrated in the thesis (Table 1).

Table 1: Overview of all the studied material, described and illustrated species are indicated in bold.

Species	Locality	Date	Method/Leg.	Identification
Tipula scripta Meigen, 1830	Oslo, Nordstrand, Ljanselva, Liadalen (59.8481ºN 10.7927ºE)	14.-28. Jul 2010	Malaise trap; Søli & Steinert	L. Boumans.
Tipula variicornis Schummel, 1833	Telemark, Porsgrunn, Brevik, Frierflauene (59.05794N° 9.66485E°)	30. Jun-27. Jul 2010	Malaise trap; Søli &Steinert	L. Boumans
Nephrotoma dorsalis Fabricus, 1781	Oslo, Nordstrand, Ljanselva, Liadalen (59.8481ºN 10.7927ºE)	14.-28. Jul 2010	Malaise trap; Søli & Steinert	L. Boumans
Epiphragma ocellare Linnaeus, 1761	Oslo, Nordstrand, Ljanselva, Liadalen (59.8481ºN 10.7927ºE)	14.-28. Jul 2010	Malaise trap; Søli & Steinert	L. Boumans
Tipula lunata Linnaeus, 1758	Telemark, Porsgrunn, Brevik, Frierflauene (59.05794 N° 9.66485E°)	30. Jun-27. Jul 2010	Malaise trap; Søli & Steinert	L. Boumans
Trichocera (trichocera)major Edwards, 1921	Oslo, Østensjø (59.87959N° 10.83502E°)	8.-28. Oct 2012	Malaise trap; G. Søli	E. Krzeminska
Trichocera (Saltrichocera) salator Harris, 1776	Oslo, Østensjø (59.87959N° 10.83502E°)	8.-28. Oct 2012	Malaise trap; G. Søli	E. Krzeminska
Trichocera (saltrichocera) rufulenta Edwards, 1938	Oslo, Østensjø (59.87959N° 10.83502E°)	8.-28. Oct 2012	Malaise trap; G. Søli	E. Krzeminska
Trichocera (saltrichocera) rufescens Edwards, 1921	Oslo, Østensjø (59.87959N° 10.83502E°)	8.-28. Oct 2012	Malaise trap; G. Søli	E. Krzeminska
Indet. Trichoceridae	Oslo, Botanical garden (59.91780N° 10.76866E°)	20. Des-15. Oct 2012	Sweep net; T. Magnussen	
Indet. Trichoceridae	Oslo, Nordstrand, Ljanselva, «Urskogen» (N°59.8541 E°10.8183)	16.-27. Apr 27. Apr-8. May 8.-19. May 2010	Malaise trap; G. Søli	
Ptychoptera minuta Tonnoir, 1919	Oslo, Østensjø (59.89129N° 10.82617E°)	15. May-20. Jun 2012	Sweep net; T. Magnussen	T. Magnussen
Ptychoptera lacustris Meigen, 1830	Bø, Hurum, Holtnesdalen (59.54037 ºN 10.42997 ºE)	9. Jun – 7. Jul 2010	Malaise trap; L O. Hansen	T. Magnussen

Slide mounting

All illustrations are based on permanent or temporary slide mounts. The illustrations were made by the use of a drawing tube attached to a Leica DMLB light microscope, with maximum magnification 63x. All material was originally stored in ethanol, and dissected in ethanol or glycerol using a Wild M8 (max magnification 50x) stereomicroscope. The descriptions are restricted to parts partly or totally sclerotized parts due to the methodology used and therefore does not allow for detailed study of soft tissue, such as muscles, accessory glands and other highly membranous structures.

Smaller individuals of Trichoceridae were slide mounted using the following method: wings, head, legs from one side and abdomen were separated from thorax. Wings and legs were transferred directly to 100% ethanol. The head, thorax with legs and the abdomen were treated with lactic acid (9%) and heated in a microwave oven for 1 minute in order to remove soft tissue. The time in the oven was adjusted according to the degree of sclerotization and pigmentation. The terminalia were then dissected from the abdomen, and in some cases further dissected by removal of the tergal structures from the genitalia in 80% ethanol. To remove all the remains of lactic acid, the parts were placed in 80% ethanol for at least 10 minutes. Subsequently, these parts were moved to 100% ethanol along with the wings for at least 10 minutes, for dehydration. Two wings, head with antennae, thorax with legs, legs and abdomen together with the genitalia were then mounted in Euparal, under separate cover slips on the slide. Thin fishing-wire or small pieces of cover glass were used to level the slip not to impact the specimen.

For the larger specimens, including the larger trichocerids, all ptychopterids and tipulids, only the terminalia where treated with lactic acid and mounted as described above. The rest of the body was stored in 80% ethanol.

The terminology mainly follows McAlpine (1981) and Sæther (1977) and are compared to the descriptions of female Mycetophilidae in Søli (1997), although the present interpretations are not always in accordance with the interpretation made by the authors.

Results

Ptychopteridae

Ptychoptera minuta

The female genitalia of *Ptychoptera minuta* (Fig. 2) consist of the fused and reduced tergites 8, 9 and 10. Tergite 8 is a heavily sclerotized curved and narrow sclerite. There is no clear boundary between tergites 9 and 10 (Fig. 2A). It is possible that tergite 10 forms the most posterior part of this compound structure, and it might also form the mid-dorsal basis of the cerci. The cerci are one-segmented, fused to about the middle of their length. The cerci are tapering and pointing downwards and the apex of each bears a small cluster of setae. Sternite 8 is present as a narrow base of the gonocoxites 8. The gonocoxites 8 are well-developed and represent the broader area of the sternal structure; most posteriorly the gonocoxite is folded inwards. The fold is densely covered with small setae, and few longer setae. Gonapophyses 8 forms a membranous sheath over both lateral sides of the gonocoxite (Fig. 2C).

Many of the structures lying posteriorly of, or around the spermathecal opening seemingly represent a very composite structure, including elements associated with segment 9 (Figs 2C, D). The composite structures are connected by membranes. The gonapophyses 9 form a flattened and partly sclerotized structure and are fused anteriorly in to a short notum. Sternite 9 is located ventrally of the gonapophyses 9 and forms an elongated, heavily sclerotized and pigmented structure. Both gonapophyses 9 and sternite 9 are closely associated with the spermathecal opening. What possibly represents the gonocoxites 9 are connected laterally to the inside of the lateral walls of sternite 8, also connected to sternite 9 and gonapophyses 9 by membranes (Fig. 2C). Close to the inner surface of sternite 8 is a sclerotized plate. The spermathecae consists of three separate spermathecal ducts, densely set with spine-like secretory cells. The spermathecal ducts end in three oval-shaped, sclerotized and pigmented seminal capsules (Fig. 2B). The transition between the duct and the seminal capsule is less pigmented.

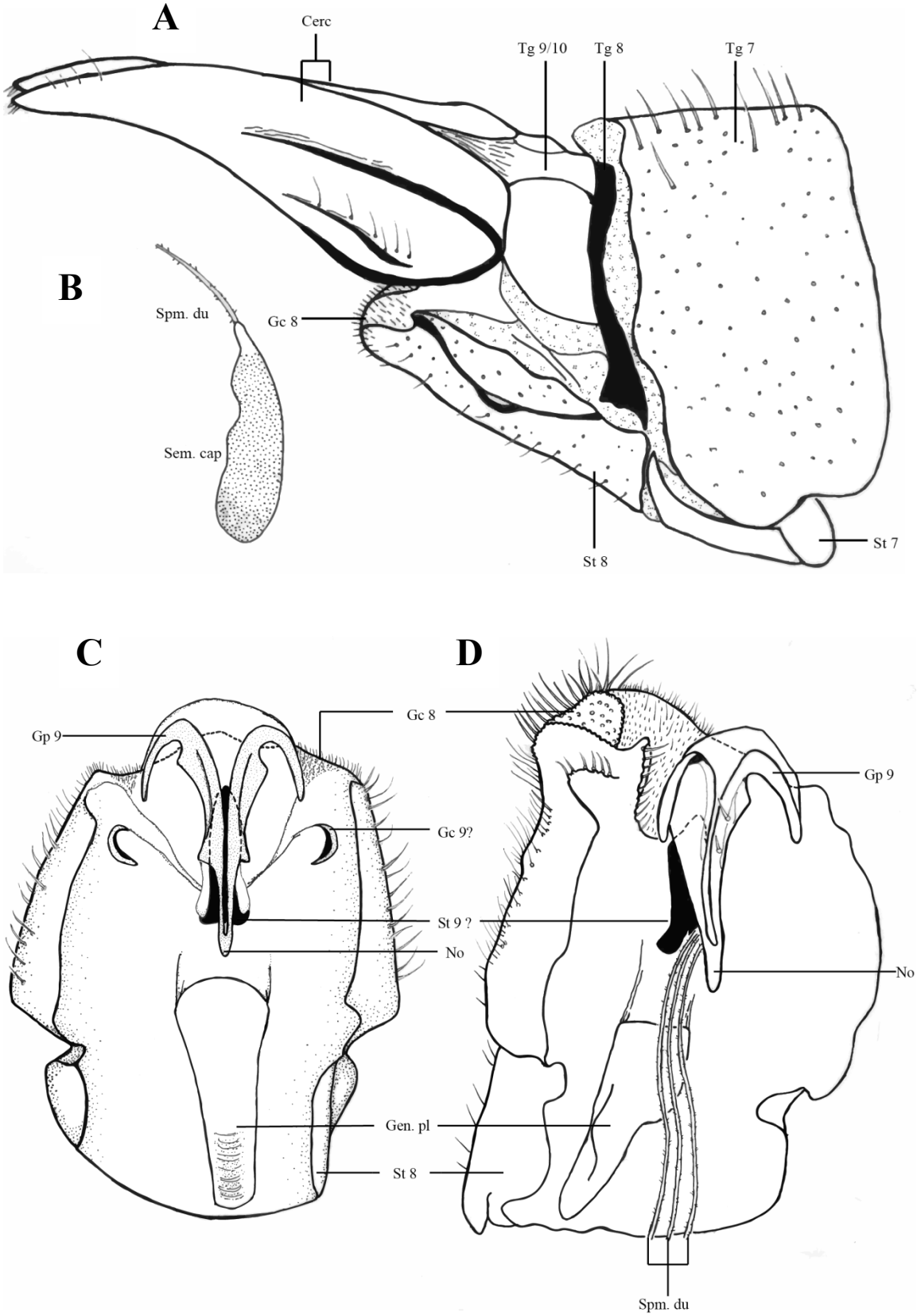

Figure 2: Female genitalia of *Ptychoptera minuta*. **–A:** Lateral view. **–B:** Seminal capsule. **–C:** Dorsal view of sternal parts. **–D:** Dorsolateral view of sternal parts**. Abbreviations:** Cerc = cerci; Gc = gonocoxite; Gen. pl = genital plate; Gp = gonapophysis; No = notum; Sem. cap = seminal capsule; Spm. du = spermathecal duct; St = sternite; Tg = tergite

Trichoceridae

Trichocera major

The female genitalia of *Trichocera major* (Figs 3 and 4) consist of partly fused tergites 8 and 9, forming a narrow anterior part, and a broader posterior part. Tergite 10 is elongated (Fig. 3B). The paired, one-segmented cerci are dorsally connected to tergite 10 with a membrane (Fig. 3B). The cerci are curved downward and each cercus has a distinct area with minute setae at the ventral base (Figs 3C, 4A). Sternite 8 is well developed and bears a pair of gonocoxites 8 caudally (Fig. 4C). Between and above the gonocoxites 8 a pair of gonapophyses 8 are present and they are equipped with setae at the posterior end (Fig. 4C). The two gonapophyses 9 meet just before the spermathecal opening and form a long and thin notum (Figs 4A, B). The gonapophyses 9 form a plate-like structure together with sternite 9, which is not separable from the gonapophyses 9, but probably makes up the area surrounding the spermathecal opening. Sternite 10 is located just dorsally of, and closely associated with the gonapophyses 9. Sternite 10 is a rounded and membranous plate, with two long apical setae. There are three spermathecae are present, the opening is located just anterior to the notum. There are three separate, weakly sclerotized, spermathecal ducts which end in round, sclerotized and pigmented seminal capsules (Figs 3A, 4A).

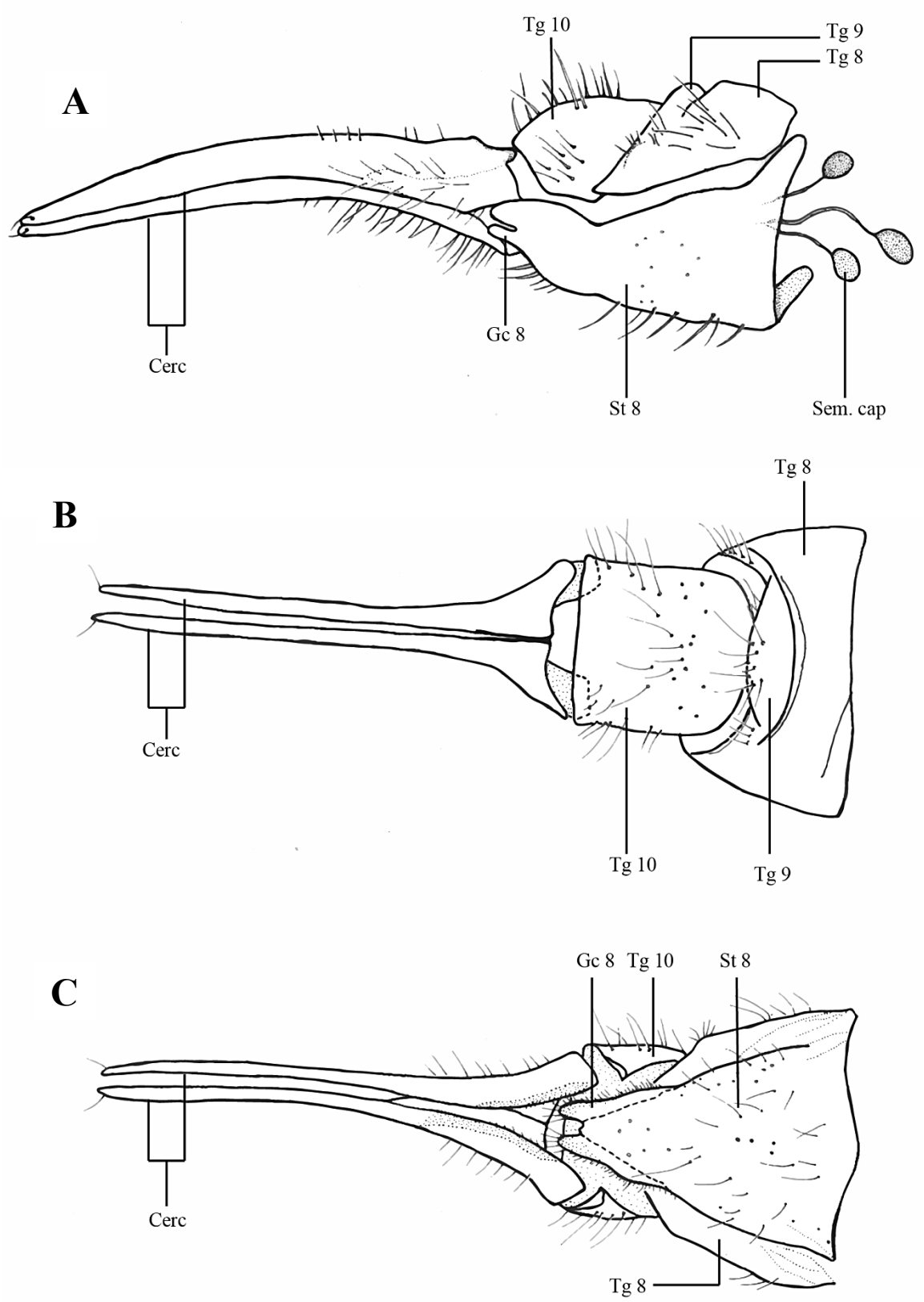

Figure 3: Female genitalia of *Trichocera major*. **–A:** Lateroventral view. **–B:** Dorsal view. **–C:** Ventral view.
Abbreviations: Cerc = cerci; Gc = gonocoxite; Sem. cap = seminal capsule; St = sternite; Tg = ternite

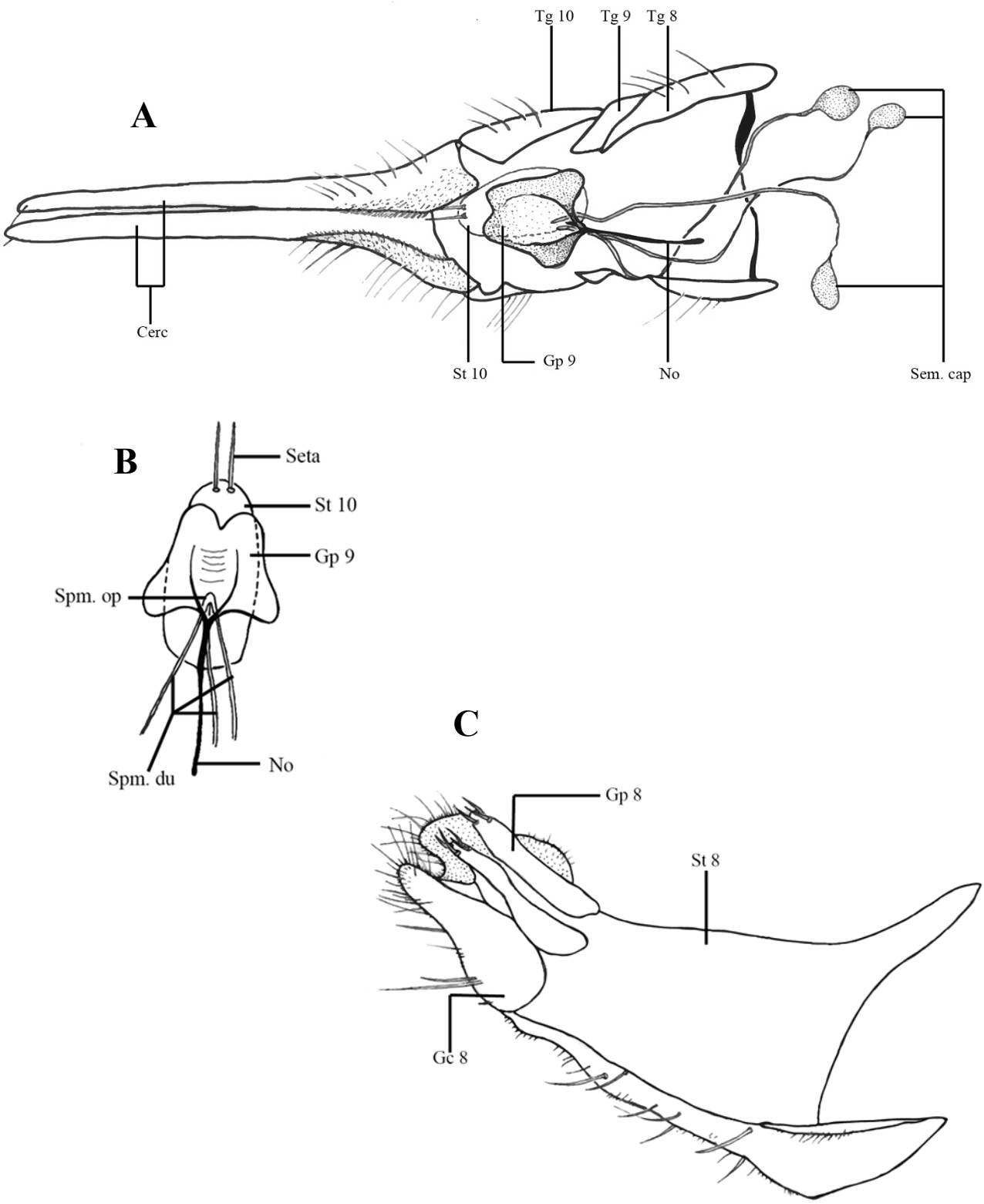

Figure 4: Female genitalia of *Trichocera major*, internal structures. **–A:** Sternal parts, ventral view. **–B:** Internal structures, ventral view. **–C**: Sternal parts, lateroventral view. **Abbreviations:** Cerc = cerci; Gc = gonocoxite; Gp = gonapophysis; No = notum; Sem. cap = seminal capsules; Spm. du = spermathecal duct; Spm. op = spermathecal opening; St = sternite; Tg = tergite.

Tipulidae

Tipula scripta

The female genitalia of *Tipula scripta* (Figs 5 and 6) have an unmodified tergite 8. Tergite 9 is reduced and holds a pair of gonocoxites 9 ventrally (Fig. 5). Tergite 10 is seemingly fused with the epiproct and extends dorsally towards the base of the cerci (Fig. 6A). The cerci are one-segmented, slightly bent upwards and the posterior-lateral sides are jagged. At the ventral side of tergite 10 is an elongated sternite 10 present. Sternite 10 is covered with setae and trichia and distinctly broadened posteriorly (Fig. 6C). The hypoproct is located between the cerci and sternite 10 (Fig. 6B). Sternite 8 and gonocoxite 8 are fused, and there is no clear separation between the two. Two caudal, elongated structures posterior of gonocoxites 8 are believed to represent gonapophyses 8 (Fig. 6C). The gonapophyses 8 have a few setae posteriorly. Sternite 9 is reduced and located posteriorly of the spermathecal opening and it is connected to the gonapophyses 9 laterally (Fig. 6C). A heavily sclerotized and pigmented genital plate is located just anterior of the spermathecal opening. It is uncertain whether or not the genital plate represents the fused gonapophyses, consequently forming a notum, or not. Due to the different outline in both sclerotization and pigmentation it is interpreted as a genital plate. There are three heavily sclerotized seminal capsules present, each having separate ducts. The three ducts fuse and form a common duct before the opening. The opening is located just anteriorly of the notum, surrounded by gonapophyses 9 and sternite 9.

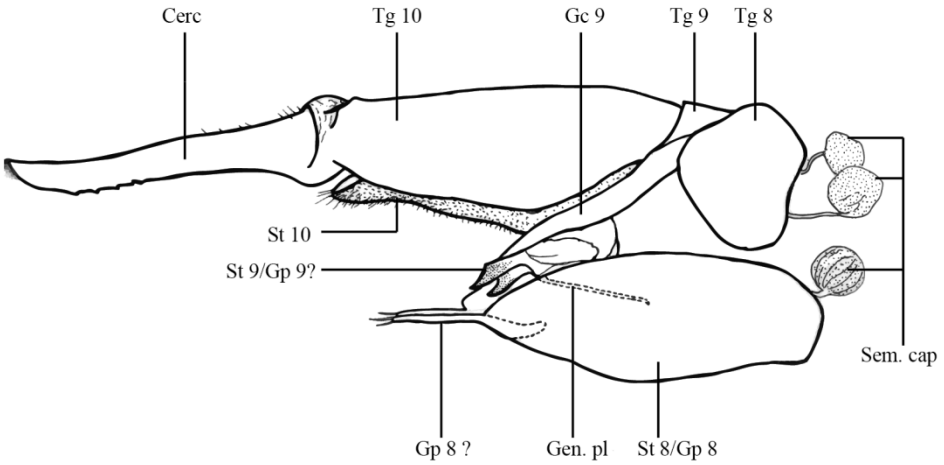

Figure 5: Female genitalia of *Tipula scripta*, lateral view. **Abbreviations:** Cerc = cerci; Gc = gonocoxites; Gp = gonapophyses; Sem. cap = seminal capsule; St = sternite; Tg = tergite

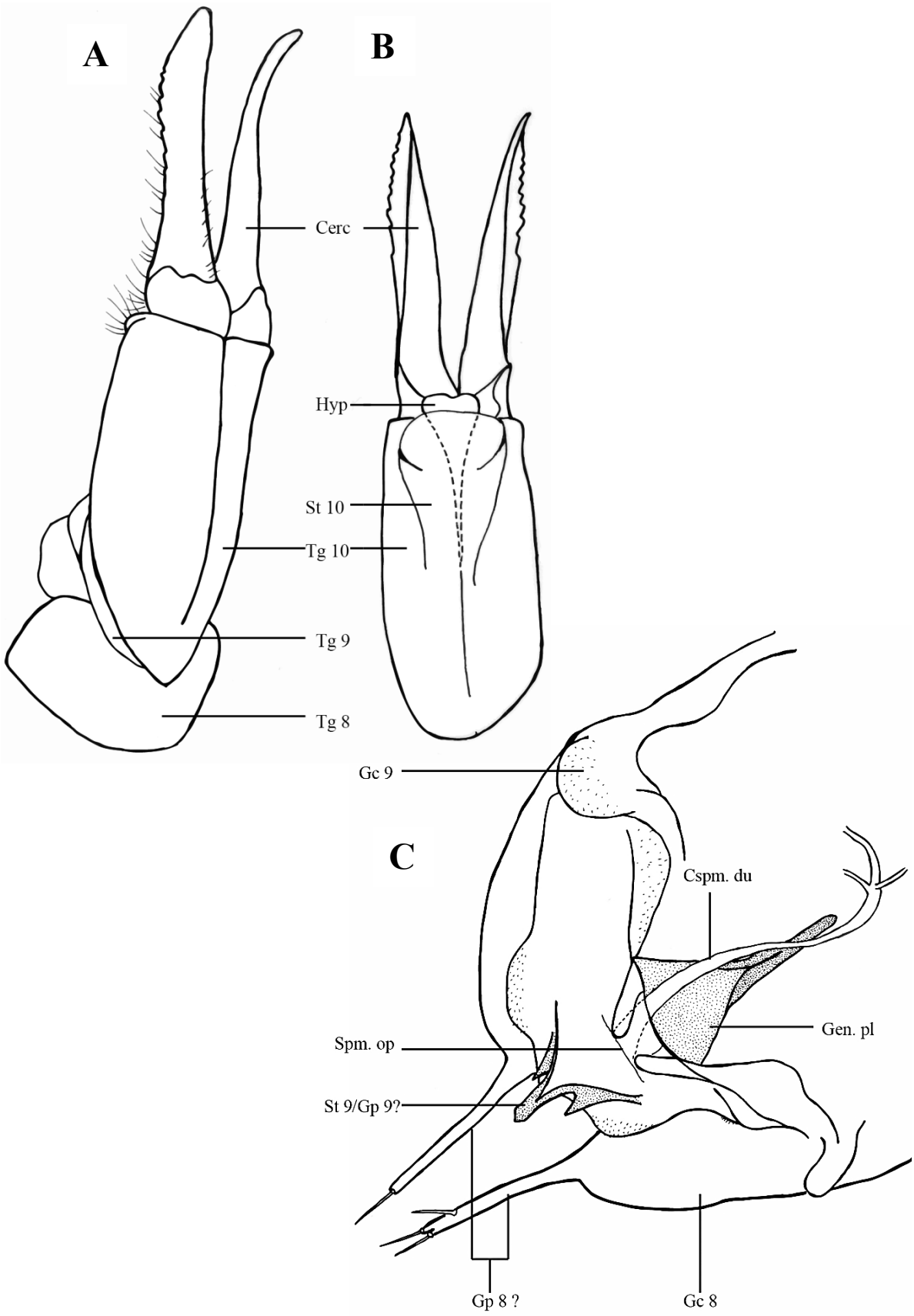

Figure 6: Female genitalia of *Tipula scripta*. **–A:** Tergal parts, dorsolateral view**. –B:** Segment 10 and cerci, ventral view. **–C:** Sternal parts, dorsal view. **Abbreviations:** Cerc = cerci; Cspm. du = common spermathecal duct; Gc = gonocoxite; Gen. pl = genital plate; Gp = gonapophysis; Hyp = hypoproct; Spm. op = spermathecal opening; St = sternite; Tg = tergite.

Tipula variicornis

The genitalia of *Tipula variicornis* (Figs 7 and 8) are composed of an unmodified tergite 8, a narrower tergite 9, and an elongated tergite 10 (Fig. 8A). Tergite 10 is possibly fused with the epiproct and this composite structure ends in a pair of cerci. The cerci are one-segmented, elongated and narrow. On the ventral side of tergite 10 the hypoproct is present, which is covered with minute setae (Figs. 7A, 8B). Sternite 10 is located ventrally to the hypoproct and is more lobe-like, with both long and short setae (Fig. 8B). Sternite 9 is reduced, but possibly fused with gonapophyses 9. Gonapophyses 9 are fused posteriorly, forming a triangular-shaped, weakly sclerotized structure (Fig. 8B) covered with minute trichia. A flattened genital plate is present anteriorly of the gonapophyses 9, with no apparent connection to the gonapophyses. Sternite 8 is fused with gonocoxites 8 posteriorly. The gonocoxites 8 serves as a basis for the blade-like gonapophyses 8. A membranous labia is present at the base of the gonapophyses 8 (Figs 7B, C). There are three weakly sclerotized seminal capsules present, each with separate ducts which fuse and form a long common duct well before the spermathecal opening. Before treated with lactic-acid the common spermathecal duct is coiled in a spiral-like manner.

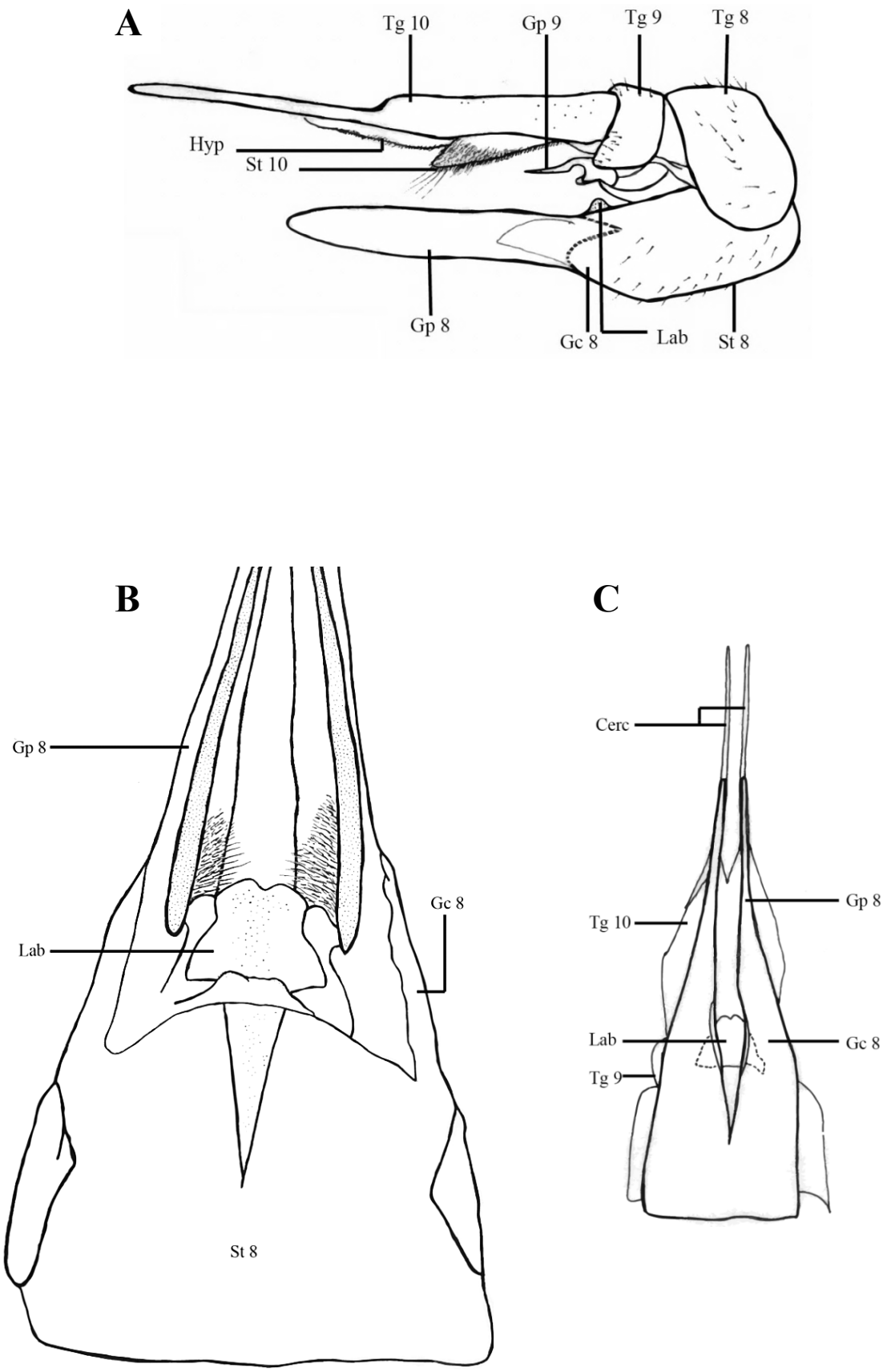

Figure 7: Female genitalia of *Tipula variicornis*. **– A:** Lateral view. **–B:** Sternal parts, dorsal view**. –C:** Ventral view
Abbreviations: Cerc = cerci; Cspm. du = common spermathecal duct; Gc = gonocoxite; Gen. pl = genital plate; Gp = gonapophysis; Hyp = hypoproct; Spm. op = spermathecal opening; St = sternite; Tg = tergite

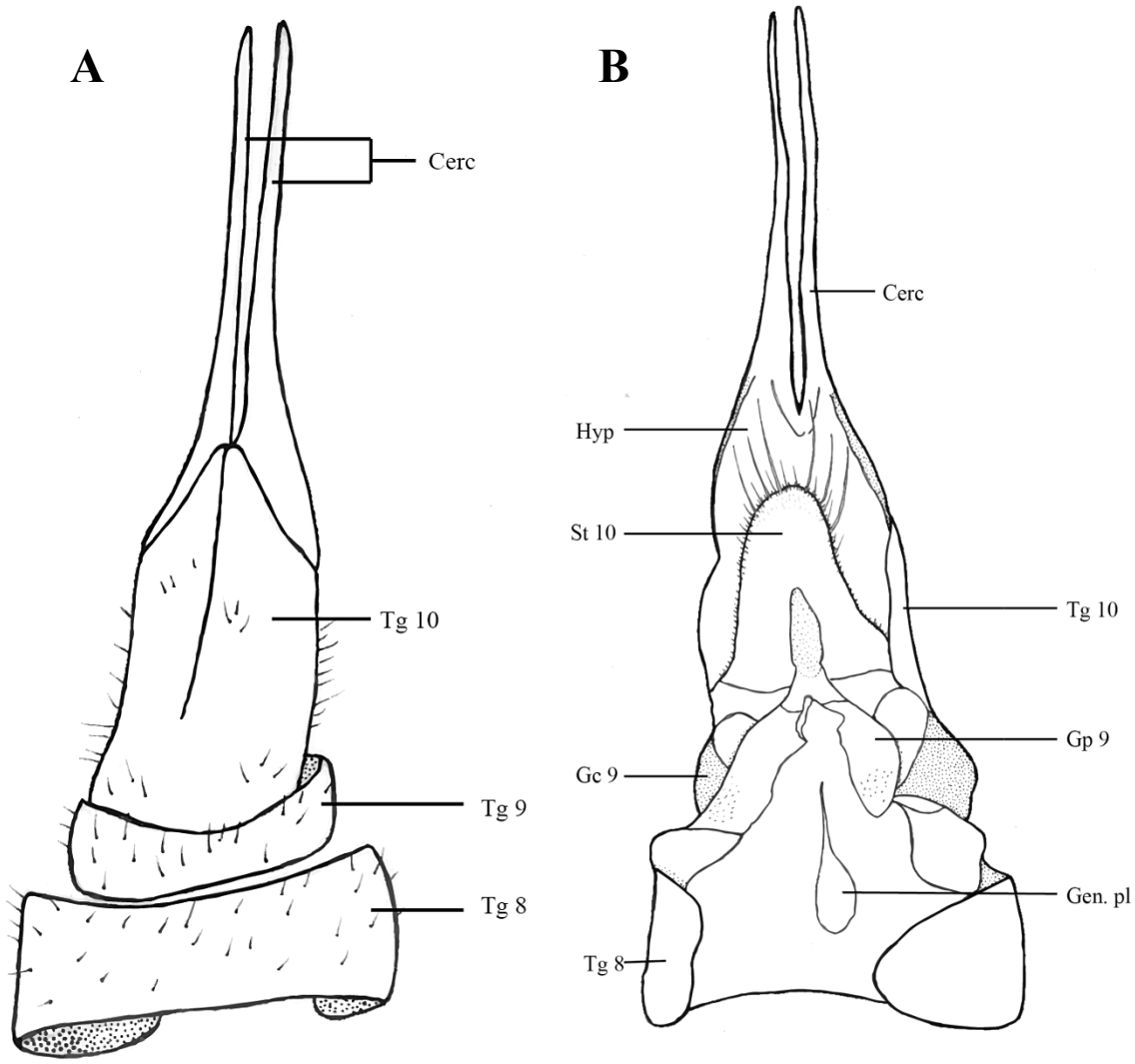

Figure 8: Female genitalia of *Tipula variicornis*. **–A:** Tergal parts, dorsal view. **–B:** Tergal parts, ventral view. **Abbreviations:** Cerc = cerci; Cspm. du = common spermathecal duct; Gc = gonocoxite; Gen. pl = genital plate; Gp = gonapophysis; Hyp = hypoproct; Spm. op = spermathecal opening; St = sternite; Tg = tergite

Discussion

The genitalia will be presented segment by segment and the variation between each of the four studied species will be commented on. The applied synonyms are listed in Appendix.

Segment 8 and spermathecae

Tergite 8: Tergite 8 is occasionally referred to as the epigyninum (McAlpine 1981). The tergite is nearly unmodified in the two studied *Tipula* species and in *Trichocera major*. In Ptychopteridae on the other hand, it is highly modified and reduced to a strongly sclerotized, bridge-like structure. Tergite 8 in *Ptychoptera minuta* is also fused with tergite 9, but separable due to differences in sclerotization. The interpretation that this structure represents tergite 8 in Ptychopteridae is in accordance with Andersson (1997) and Peus (1958). The reduction and outline of tergite 8 is one of the most important differences between Ptychopteridae and the two other studied families.

Sternite 8 and gonocoxites 8: The combined sternite 8 and gonocoxites 8 is commonly referred to as the hypogynium (McAlpine 1981). All studied species have distinct and well developed gonocoxites 8, though varying in shape. The most conspicuous outline of this combined structure is found in *P. minuta* where the sternite is broad and covers large parts of the genital chamber. This outline could be a consequence of the reductions observed in the tergal structures, which have caused the sternite 8 to cover most of the genital chamber. By studying illustrations in Andersson (1997) one can see that the special shape of sternite 8, which he refers to as the subgenital plate, is not unique for *P. minuta* but also exists in other *Ptychoptera* species. In Trichoceridae sternite 8 is much less concave and broader anteriorly. Posterioriorly tergite 8 ends in gonocoxites 8, each forming a small lobe at the apex of sternite 8. The two *Tipula* species studied have a quite similar outline of sternite 8 and gonocoxites 8. In *Tipula variicornis* sternite 8 has a caudal prolongation termed the labia (Sæther 1977). This structure is not found in *Tipula scripta*. The gonocoxites 8 in *T. variicornis* are present as two medially connected triangular-shaped sclerotizations, with a clear split on the ventral side of the sternite 8/gonocoxite 8. In comparison this split is not so apparent in *T. scripta*.

Gonapophyses 8: McAlpine (1981) denotes this paired structure hypogynial valves. The hypogynial valves are defined in Crampton *et al.* (1942) as being appendages of sternite 8, homologous to the gonapophyses 8. This is in accordance with the interpretation of Rees and

Ferris (1939) in their description of Tipulidae. Frommer (1963) and Byers (1961) disagree with this view and consider the hypogynial valves to be sternal extensions. de Jong (1997) in his study of intersexes and homology of genital structures in Tipulidae, found that the posterior extensions of sternite 8 in males are homologous to the female hypogynial valves (gonapophyses 8). *T. variicornis* has the most prominent and well developed gonapophyses 8, which are long and bladelike, with an area covered with setae on the internal basis. In comparison, *T. scripta* has small, spine-like gonapophyses 8, which is much shorter than the cerci. According to Alexander (1920) the larvae of *T. scripta* live in earth and decaying plant material in woods, while the larvae of *T. variicornis* are associated with freshwater. These two larval habitats might be reflected in the differences in the outline of the ovipositor, because the substrate for egg-laying differs between the species. In this study the gonapophyses 8 in Tipulidae are considered homologous to gonapophyses 8 in Trichoceridae, in accordance with McAlpine (1981). Dahl and Krzeminska (1997) considered the hypogynial valves (here termed gonapophyses 8) of Trichoceridae to be remnants of the midventral parts of segment 9, and based their interpretations on the study of Dahl (1980). The present results differ from their interpretation, as the gonapophyses 8 in both families are here considered to be homologous, and derived from segment 8 and not segment 9. In *T. major* the gonapophyses 8 are distinct, but smaller and less prominent than in the *Tipula*-species and the gonapophyses 8 do not exceed the length of the gonocoxites 8. The gonapophyses 8 are reduced in Ptychopteridae, and might only be present as membranous sheaths covering the dorsal side of the gonocoxite 8. Hence, well-developed gonapophyses 8 is common for the studied species of Tipulidae and Trichoceridae.

Spermathecae: The spermathecae are the sperm storage organ, derived from segment 8 (McAlpine 1981). As already mentioned the spermathecae are primitively composed of three seminal capsules, each with separate ducts and openings (Downes 1968). All species studied have three seminal capsules present, but one capsule is known to occur in some species of Trichoceridae (Krzeminska 2001). In both Trichoceridae and Ptychopteridae there are three separate ducts which do not fuse before they open into the genital chamber. The spermathecal ducts of *P. minuta* are covered with small spine-like cells as described in Sæther (1977). However, this is not found in Trichoceridae and Tipulidae. In Tipulidae the ducts meet well before the opening, and the length of this common spermathecal duct varies between *T. scripta* and *T. variicornis*. In the latter, it is very long and curled, while in *T. scripta* short and straight.

Segment 9

Tergite 9: In Ptychopteridae tergite 9 is fused with tergite 8 and tergite 10 to form a common sclerite between tergite 7 and the cerci. There is no clear separation between tergite 9 and tergite 10, and they are seemingly completely fused, Andersson (1997) interprets tergite 10 to be lost in Ptychopteridae. In *T. scripta* tergite 9 is reduced and only visible as a narrow sclerite dorsally, but extends into the well-developed gonocoxites 9 laterally, which makes the structure more recognizable. In *T. variicornis*, tergite 9 is apparent and only slightly reduced, but also in this species the gonocoxites 9 are closely associated to the tergite 9. In Trichoceridae, tergite 9 is reduced and partly fused with tergite 8, but in other species tergite 9 might also be fused with tergite 10 (pers. obs.). Dahl (1980) in her study of postembryonic organization in the larvae of Trichoceridae, concluded that no tergite 10 is present in Trichoceridae and that the apparent structure was only a superficial division of tergite 9. The present findings and interpretations, based on the observations of the several species of Trichoceridae, do not agree with her interpretation.

The inner genital structures: Sternite 9 and the gonapophyses 9 are the most modified structures in all the families studied. They are closely associated with the spermathecal opening and the genital chamber. In close affiliation to sternite 9 is an anterior genital plate, and a pair of gonapophyses 9, located more posteriorly. These structures are frequently denoted as the genital fork or furca in Tipulidae (Byers 1961) and other dipteran families (Tuxen 1970). The gonapophyses 9 are often described as sternite 9 (see Appendix) in Tipulidae (Rees and Ferris 1939; Frommer 1963), Trichoceridae (McAlpine 1981) and Ptychopteridae (Peus 1958; Andersson 1997). The term notum is here applied to what is commonly referred to as the vaginal apodeme, i.e. the vaginal apodeme is an anterior extension of the gonapophyses 9, as described in Sæther (1977). If the vaginal apodeme not appear to be an extension of the gonapophyses 9, the term genital plate is used. This is done to express the uncertainty about whether or not they represent homologous structures. As previously mentioned, the gonapophyses 9 are well developed in all the studied families, and located posterior to or surrounding the spermathecal opening. In both *T. major* and *P. minuta* the gonapophyses are partly fused and form a flat, plate-like structure. This plate has several membranous parts in *P. minuta*, which is not found in *T. major*. In the *Tipula* species the gonapophyses 9 are in close association with the gonocoxites 9. Sternite 9 is most likely strongly reduced and fused with the gonapophyses 9 in both Trichoceridae and Tipulidae. This situation differs from that in Ptychopteridae, where a structure tentatively termed sternite

9 is present dorsally of the gonapophyses 9. It is uncertain whether or not this represents a true sternite 9. Trichoceridae has the most elongated and prominent notum. In *P. minuta*, a very short notum is present in addition to a genital plate located just dorsally of sternite 8 in the floor of the genital chamber. Both Peus (1958) and Andersson (1997) refer to what is here termed the genital plate as the anterior vaginal apodeme, or just vaginal apodeme. Both *T. variicornis* and *T. scripta* has a sclerotized plate located in the same area as the genital plate in *P. minuta*, here termed genital plate, as no apparent connection to gonapophyses 9 has been observed.

Gonocoxites 9: The gonocoxites from segment 9 is distinct in both *Tipula* species. Rees and Ferris (1939) in their description of *Limonia sciophila* Alexander, 1958, denoted the same structure as the coxopodite (=gonocoxite). The structures are here termed gonocoxites 9 due to the close affiliation to the gonapophyses 9. In Trichoceridae the gonocoxites 9 could not be identified and are thus regarded as reduced or possibly fused with tergite 9. Neither in Ptychopteridae distinct gonocoxites 9 could be found, but they may be fused with the inside wall of sternite 8. Andersson (1997) presents an illustration of *Ptychoptera contaminata* Linnaeus, 1758, with structures likely to represent the gonocoxites 9. *P. minuta* shows structures similar to these, but less pronounced.

Segment 10 and proctiger

Tergite 10: As previously mentioned tergite 9 and tergite 10 are here interpreted as either being completely fused (with no clear separation), or alternatively that tergite 10 is lost in Ptychopteridae. This is not in accordance with Rozkosny (1997) and Peus (1958) which considered the basal part of the cerci to be tergite 10, since the cerci are fused to about the middle of the length. *Trichocera major* is interpreted as having an elongated tergite 10 which contradicts Dahl (1980), who considers tergite 10 to be lost in Trichoceridae. In both *Tipula*-species, tergite 10 is elongated and apparent.

Sternite 10: Sternite 10 in Tipulidae is termed infra-anal plate (Frommer 1963), or alternatively post-genital plate (Sæther 1977). In Trichoceridae sternite 10 is referred to as vaginal plate (Dahl 1980) or supravaginal plate (Dahl and Krzeminska 1997). In both *Tipula scripta* and *T. variicornis*, sternite 10 is a distinct lobe-like structure located ventrally of tergite 10 and covered with fine setae. In Trichoceridae, tergite 10 is reduced and membranous, but with two distinct setae apically, located at the same level as the

gonapophyses 9. In Ptychopteridae both tergite 10 and sternite 10 appear to be strongly reduced and thus difficult to recognize. In *Ptychoptera contaminata* Peus (1958) suggests that a small sclerotization ventrally, at both sides of the cerci, represents the remains of sternite 10. This structure was not detectable in *P. minuta*. The well-developed sternite 10 is unique to Tipulidae when compared to Trichoceridae and Ptychopteridae. The complete reduction of sternite 10 further separates Ptychopteridae from Trichoceridae.

Cerci: What is here termed cerci are considered to be homologous structures in the studied families. This contradicts the findings of Dahl (1980) who considers the cerci of Trichoceridae and Tipulidae to be derived from segment 9 and homologous to the male gonocoxite and gonostylus, but observations made by de Jong (1997) contradicts this result. Based on his study of intersexes in Tipulidae, he homologizes the gonocoxite and the gonostylus with the processes of sternite 9 (the hypogynial valves). The cerci in all the studied species are one-segmented and tapering. In fact one-segmented cerci is a character used to group Tipulidae and Trichoceridae together in the suborder Tipulomorpha (Yeates and Wiegmann 1999). This is considered a derived state in contrast to the two-segmented cerci found in most other families of the lower Diptera (i.e. Mycetophilidae). Sæther (1977) described the cerci of Ptychopteridae as two-segmented with the last segment reduced; the small apical setae were interpreted as the reduced second segment. According to observations made in this study this is not the case, an interpretation that is in accordance with Peus (1958) and Andersson (1997). The trichocerid cerci are long and tapering with a ventral groove. The ventral parts of the cerci in Tipulidae on the other hand are covered by the hypoproct and apical part of sternite 10. The cerci in Tipulidae are straight or bent slightly upwards, while the cerci in Trichoceridae points downwards. Most of the families in lower Diptera place their eggs in aquatic or semiaquatic environments, hence the substrate in which the female lay their eggs is important concerning the outline of the genitalia (Hünefeld *et al.* 2012). It might therefore bee that the special outline of the cerci is related to the substrate for egg laying in the studied families. Although they place their eggs in moist substrate, an effective ovipositor can aid in burying the eggs in e.g. silt, detritus or mud, which may shield and protects the eggs.

Epiproct: The epiproct is not observed as a clearly recognizable structure in any of the species studied. This is not surprising as it is reduced in female Diptera in general (McAlpine 1981).

It might be fused completely with tergite 10 in Tipulidae, inferred from the well-developed hypoproct situated ventral of tergite 10 and the cerci.

Hypoproct: The hypoproct is present in both *T. scripta* and *T. variicornis*. In *T. scripta* the hypoproct is distinct and present just dorsal of sternite 10, which is heart-shaped apically. In *T. variicornis* the hypoproct is fused to the underside of tergite 10 and the basis of the cerci. In *P. minuta* and *T. major* no hypoproct could be detected.

Conclusive remarks

The comparison of the female genitalia in the selected families of lower Diptera, clearly demonstrates the complexity in female genitalia, but also that it is possible to homologize between structures. The observed complexity was even higher than expected. The internal genital structures, which include the structures derived from segment 9, are the most modified in all studied species, and they are homologized largely based on their position in relation to the other structures. It was especially difficult to homologize the complex internal structures of *Ptychoptera minuta* to those found in Tipulidae and Trichoceridae. What is also unique to Ptychopteridae is the strong reduction of the dorsal sclerites. In addition, but presumably related to this reduction, are the well-developed sternite 8 and gonocoxites 8. This might reflect the isolated systematic position of the family Ptychopteridae, as suggested by Bertone (2008).

All the three studied families have distinct, well-developed and tapering cerci. Furthermore, they all have a sternite 8 with a pair of well-developed gonocoxites 8 caudally, with no apparent transition between the sternite and the gonocoxite. Tergites 8, 9 and 10 showed modification and reduction in all families, most pronounced in Ptychopteridae. Tipulidae and Trichoceridae both have well-developed gonapophyses 8, which are reduced and membranous in Ptychopteridae. Compared to Tipulidae, Trichoceridae has a rather simple and uniform outline of the internal structures. A unique feature of *Trichocera major* is the fused tergite 8 and tergite 9, with a traceable fusion line. Tipulidae are unique in relation to the two other studied families, in having a clearly recognizable hypoproct, a well-developed tergite 10 and well-developed gonocoxites 9.

The phylogeny of the lower Diptera is still no satisfactory resolved, and for many taxa good diagnostic characters and synapomorphies are lacking. In this aspect it is important to search for new characters. The achieved results suggest that a more thorough study of female genitalia that include more genera and species within each family are likely to give us a broader understanding of the evolution of the female genitalia in Diptera.

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<u>Manuscript 2:</u> Interspecific variation in female genitalia in four species of *Allodia* Winnertz (Diptera: Mycetophilidae) identified through DNA barcoding

Abstract

The female terminalia of four species belonging to the genus *Allodia* Winnertz, 1863 are described and illustrated, *A. lugens* Wiedemann, 1817, *A. zaitzevi* Kurina, 1998, *A. pyxidiiformis* Zaitzev, 1983 and *A. tuomikoskii* Hackman, 1971. Short descriptions of the general morphology are given. The females are associated with already identified males through DNA barcoding. It is demonstrated that the genital structures contains the most trustworthy characters to separate the species, while other characters such as body size, coloration of abdomen and the chaetotaxy of the head and the thorax are highly variable within species.

Introduction

Differences in the female genitalia of closely related species of Diptera are generally understudied, which to a large extent is due to vague differences in the genital structures. In contrast, the male genitalia are often described in detail because of the high interspecific variation in most groups (McAlpine 1981). Male genital structures are therefore used to separate closely related species. For some groups, this has led to an almost exclusive use of males in identification keys, which makes identification of females difficult. The development of molecular methods, such as DNA barcoding, makes it easier to associate males and females, and thus facilitates the study of both sexes within a species (Hebert *et al.* 2003).

The genus *Allodia* Winnertz, 1836, belongs to the family Mycetophilidae, and is placed in the tribe Exechiini. Exechiini consists of 19 genera altogether, although the relationship between the genera is not fully resolved (Rindal and Søli 2006; Rindal *et al.* 2007). The genus includes about 50 described species worldwide (Kjærandsen 2007), of which most are described from the Palearctic region (Bechev 2000). In Norway 15 species have been recorded (Gammelmo and Søli 2006), with an additional two species which are not yet formally described (Søli and Rindal 2012). According to Zaitzev (2003) adult *Allodia* are common and often very abundant in forest ecosystems during spring and early summer. *Allodia* was divided in two subgenera by Tuomikoski (1966), the nominotypical *Allodia* and *Brachycampta* Toumikoski, 1963. The species studied here belong to the subgenus *Allodia*. The separation of the two subgenera is largely based on male genital characters (Kjærandsen 2007). In the key presented by Zaitzev (2003), separation of the species of *Allodia* is almost exclusively based on male genitalia, this is in accordance with the observations made by Kurina (1997). Females of the species included in *Allodia* have not previously been described, as for many other species within Mycetophilidae.

In this study, females in the genus *Allodia* are associated with males through DNA Barcoding. This has previously proven to be a successful method for association of the sexes within Mycetophilidae (Kurina *et al.* 2011). The female genitalia and general morphology of four species within the genus will be described with the intention to document differences in the female genitalia at the species level.

Material and Methods

Material

The studied material all originate from one large sample collected by the use of sweep net between boulders and crevices along Gåppaelva, Alta (N70.02786 E23.39476) on June 13, 2010. Alta is located in Finnmark county in northern Norway. The material was collected by Geir Søli. The males from the same sample have already been identified and published, see Søli and Rindal (2012), as part of a large faunistic study, funded by the Norwegian Taxonomy Initiative (Artsdatabanken) (Ekrem *et al.* 2012). All the studied material was preserved in 80 % alcohol.

Four species from the genus *Allodia* were chosen for the morphological study: *Allodia pyxidiiformis* Zaitzev 1983, *Allodia tuomikoskii* Hackman 1971, *Allodia zaitzevi* Kurina 1998 and *Allodia lugens* Wiedemann 1817.

Methods

All females belonging to the genus *Allodia* were sorted out following the identification key provided by Søli *et al.* (2000). The females were divided into groups based on general morphology, such as color, external genitalia, size and chaetotaxy (arrangement of setae). A total number of 57 individuals were selected, representing 16 morphological groups. Following the procedure described in the Microplate Submission Package from Canadian Centre for DNA Barcoding, University of Guelph (CCDB), one leg from each of the 57 individuals were removed and placed in sampling wells, which were prefilled with 30 µL ethanol (96%) on a microplate. The samples were then shipped to CCDB for sequencing. The sequences and meta-data have been deposited in the Barcode of Life Data Systems (BOLD) (Ratnasingham and Hebert 2007) in the project: NorBOL- Fungus gnats [NOMYC] and will be made available after publishing.

The obtained COI sequences were aligned together with COI sequences from males ("Mycetophilidae of Finnmark"[MYCFI]) retrieved from BOLD (Ratnasingham and Hebert 2007) in the program MEGA 5 (Tamura *et al.* 2011), with the method MUSCLE. The Neighbor-joining analysis was performed using MEGA 5 and the model-test option found the

best suitable substitution model for the sequences, hence the Tamura-Nei algorithm (Tamura and Nei 1993) was applied. Bootstrap values were calculated, using 1000 iterations.

Based on the results retrieved from CCDB, four species (*A. zaitzevi, A. pyxidiiformis*, *A. tuomikoskii* and *A. lugens*) were chosen for the comparative morphological study. In addition 8 individuals were sequenced at the Natural History Museum of Oslo (NHM). Four of the 8 specimens were possible *A. pyxidiiformis* and four *A. tuomikoskii*, determined based on the results of the DNA barcoding at CCDB in combination with the morphological study.

DNA was extracted from the leg of the 8 individuals using the Tissue DNA Spin Protocol of the E.Z.N.A® Tissue DNA Kit, with following modifications: One leg from each individual was minced together with 200 µL TL Buffer in step 1, step 2 and 4 were carried out and the samples were then incubated and the lysis proceeded overnight. Subsequently steps 4 – 13 were performed. The elution step (step 13) was carried out twice, first time with 150 µL (70˚C) elution buffer and the second time with 50 µL (70°C) elution buffer, which led to a total amount of 200 µL.

A fragment of 648 base pairs (bp) from the mitochondrial cytochrome oxidase subunit I (COI) was amplified and sequenced using the primer pair LCO 1490_t1 (forward) and HCO 2198_t1 (reverse).

LCO1490_t1: 5'-TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG-3'
HCO2198_t1: 5'-CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA-3'

These primers are modified universal Folmer primers (Folmer *et al.* 1994), termed tailed Folmer set. The primers have been successfully used in the BOLD (NorBOL) project Fungus gnats [NOMYC] in 2009.

The PCR setup for a total reaction volume of 12.5 µL included; 6.54 µL dH_2O, 1.25 µL Buffer, 0.63 µL $MgCl_2$, 1.00 µL dNTP, 0.25 µL of each primer, 0.075 µL polymerase and 2.5 µL DNA. The PCR amplifications were performed using the Platinum® *Taq* DNA Polymerase of Invitrogen.

A thermal cycler was used for the PCR, with the following protocol: Initial denaturation 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 sec, synthesis at 51°C for 30 sec and elongation at 72°C for 1 min, with final elongation step at 72°C for 10 min.

To control, check for product and length of DNA sequences, gel electrophoresis was performed using 1% agarose gel, stained with 2 µL GelRed™ Nucleic Acid Gel Stain (Biotium). A Fast Ruler™ Low range DNA Ladder (Fermentas®), with length 50-1500 bp was used. 2.5 µL amplified DNA plus 2.5 µL loading buffer were added to the gel wells. The electrophoresis ran at 85 V for 20 min.

ExoSAP-IT® (Affymetrix® (USB Products®)) was added to the samples with amplified DNA in order to remove excess dNTPs and primers before sequencing. 0.4 µL of 10 times diluted ExoSAP-IT® was added per 1.0 µL of PCR product. The samples were incubated at 37°C in 45 min followed by 80°C for 15 min in a thermal cycler. The 8 samples were sequenced at StarSEQ® GmbH, Mainz, Germany.

Sequences retrieved from StarSEQ® were edited using the program CodonCodeAligner version 3.7.1 (CodonCode Core, Dedham, MA, USA). The program was used to cut (0,05%) and make a consensus sequence from the forward and reverse sequence for each sample. Each of the 8 consensus sequences was subsequently inspected manually, to check for ambiguous peaks and stop codons. Each sequence was blasted through all barcode records in BOLD and identified through the BOLD-identification option in a taxon ID-tree (Ratnasingham and Hebert 2007) in order to test if the edited sequences clustered within the already identified males from the same species (see above).

Pictures of each individual were taken with a Nicon D3100 camera and these pictures were used to make figure 3. Using a Wild M5 stereomicroscope (maximum magnification 50x), the terminal part of the abdomen from 1 to 4 individuals per species was removed and macerated in lactic acid, using a microwave oven for about 40 seconds. The rest of the individual were stored in 80% ethanol, at 4°C. The terminalia was transferred to glycerol on a microscope slide. The drawings were made by the use of a drawing tube attached to a Leica DMLB light microscope, with 40 times magnification (maximum magnification 63x).

The genitalia are described in detail for each species. In the description of the general morphology only the most important characters are highlighted and the description should not be regarded as a species description. Body and wing length were measured on individuals preserved in alcohol. Five individuals per species were measured and the range is given for each species, to highlight the intraspecific variation. The general terminology follows McAlpine (1981); for the terminalia, the terminology is in accordance with Søli (1997).

Results

Association of females through DNA Barcoding

The results retrieved from CCDB showed that all the specimens in the 16 morphological groups clustered together with the males of seven *Allodia* species already sequenced in BOLD (Fig. 1). The seven species are: *A. tuomikoskii*, *A. anglofennica* Edwards, 1921, *A. truncata*, *A. lugens*, *A. pyxidiiformis*, *A. septentrionalis* Hackman, 1971 and *A. zaitzevi*. In the Neighbor-joining analysis the seven species clusters, including both sexes, have high bootstrap support (99%).

All of the possible *A. pyxidiiformis* and *A. tuomikoskii* sequenced at NHM turned out to be correctly designated, after identification in BOLD (not included in Fig. 1).

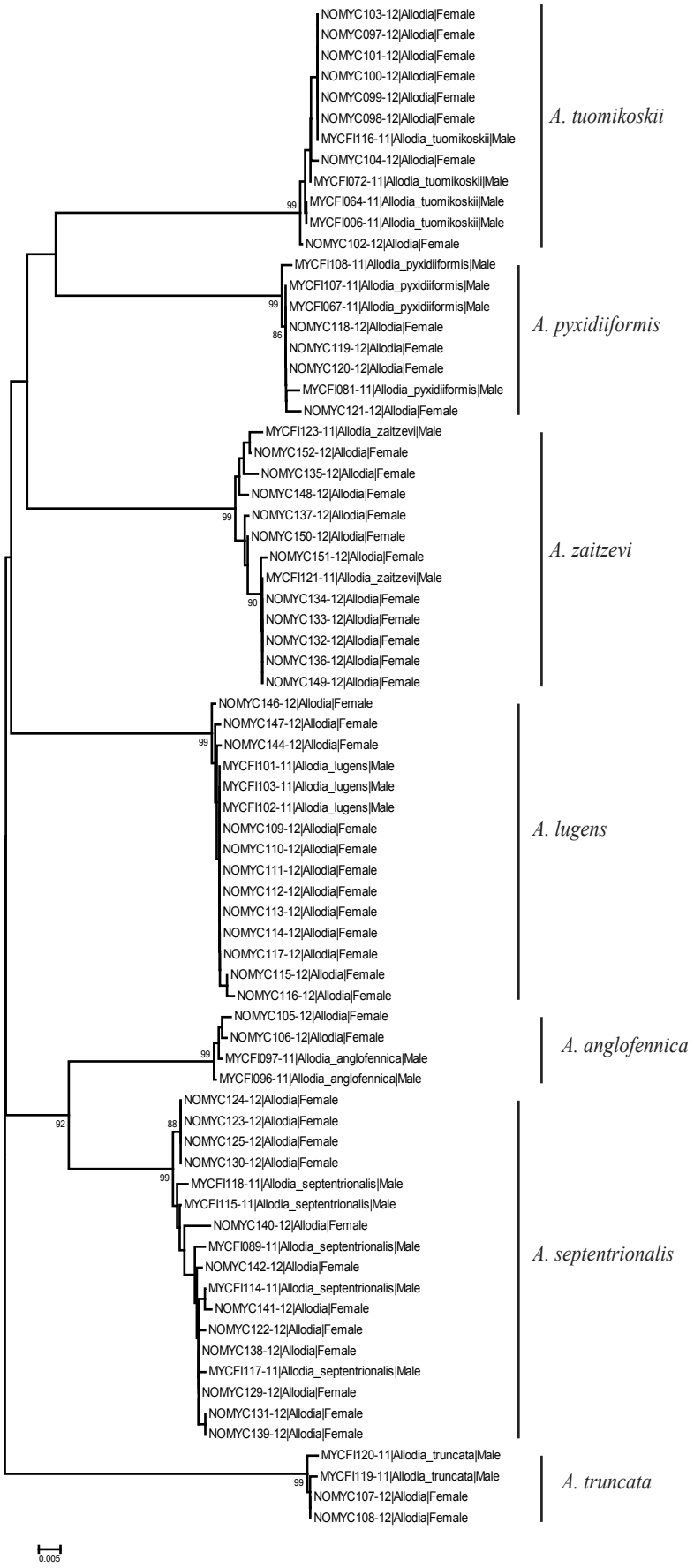

Figure 1: Clustering of the sexes. Neighbor-joining analysis with the substitution model Tamura Nei + Gamma, based on 73 sequences of the COI gene, from the merged projects "Mycetophilidae of Finnmark"[MYCFI] and "NorBOL-Fungus gnats" [NOMYC], retrieved from BOLD (Ratnasingham and Hebert 2007). Bootstrap values (1000 iterations) over 75 shown at each node.

Morphology

The morphological descriptions are given separately for each species, including size, abdominal coloration and outline of the genitalia. The chaetotaxy of head and thorax were highly variable within species and are therefore not included in the description. The terminalia of *Allodia zaitzevi* was selected as an example of the general morphology of the terminalia, and have been illustrated from the ventral, dorsal and lateral view, while the three other species are illustrated in lateral view only.

The female terminalia of all the studied species consist of an unmodified and bare tergite 8. Tergites 9 and 10 are strongly reduced and form a membranous lateral area at the basis of the cerci, but are dorsally visible as a narrow area extending posteriorly into two lobes, which form the basis of the cerci (see Fig. 2A). Dorsally, between the cerci and posterior parts of tergite 9/10, a membranous structure, which might represent the epiproct is present (Fig. 2B). The cerci are one- or two-segmented, with several distinct setae. Sternite 10 is well developed and seemingly fused with the membranous gonapophyses 9 ventrally. The seemingly combined sternite 10 and gonapophyses 9 form a prolonged, triangular shaped structure. The two spermathecal ducts open on the ventral side of gonapophysis 9, but the opening is difficult to trace. The seminal capsules are highly membranous and therefore impossible to visualize with the applied methodology (Søli 1997). Sternite 8 bears a pair of well-developed gonocoxites 8 caudally (see Fig. 2B). The gonocoxites each have one pronounced large seta apically. Sternite 8 ends in well-developed, membranous labia, which are covered with minute trichia. The labia extend posteriorly in the area between the gonocoxites 8. In all the studied species the genitalia are partly retracted into segment 7.

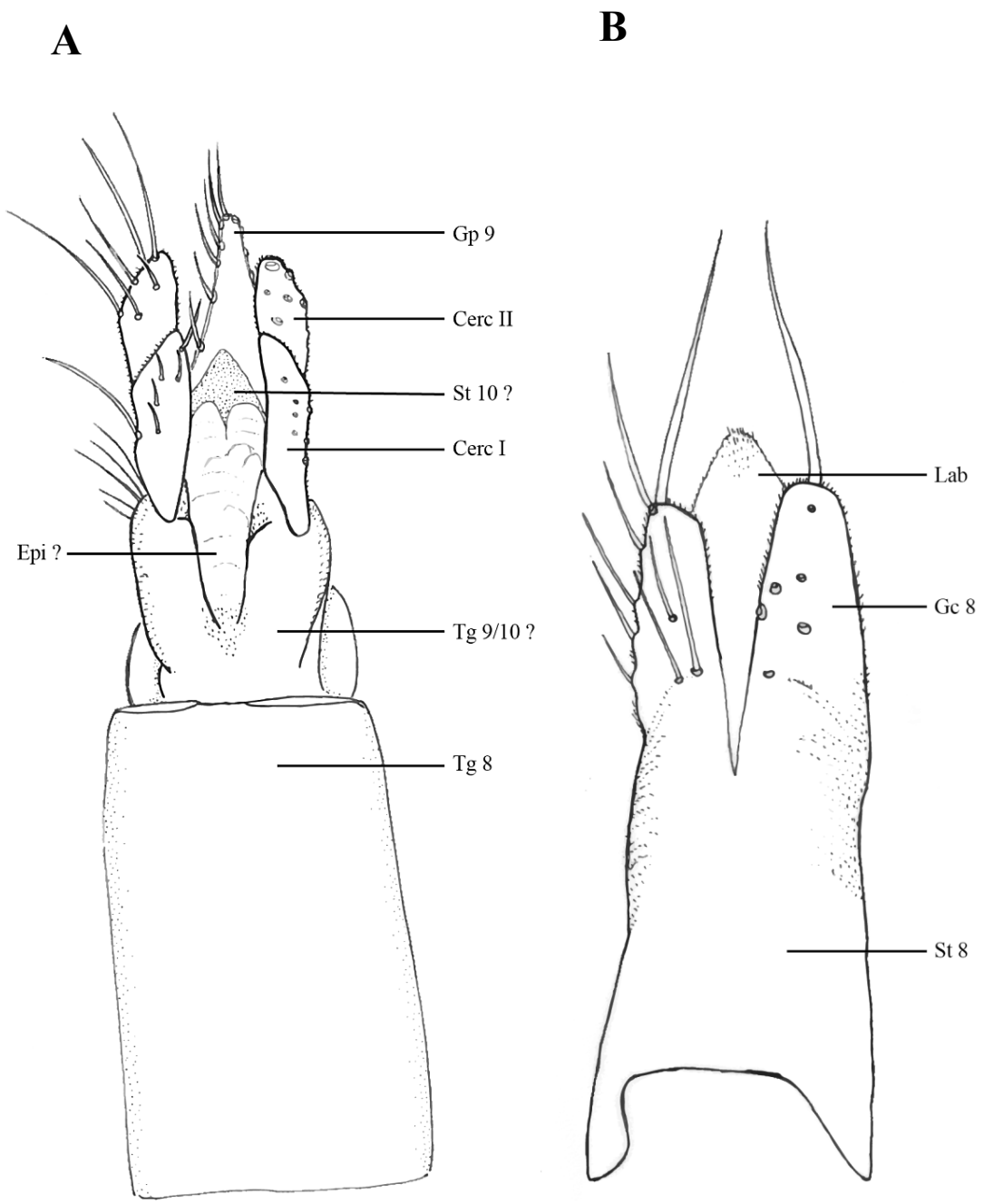

Figure 2: The female terminalia of Allodia zaitzevi, **-A:** Dorsral view, **-B:** Ventral view. **Abbreviations:** Cerc = cercus; Epi = epiproct; Gc = gonocoxite; Gp = gonapophysis; Lab = labia; St = sternite; Tg = tergite

Allodia zaitzevi Kurina, 1998

(Figs 2A, 2B, 3A and 4A)

Size: Total length ranging from 3.68 mm to 4.23 mm. Wing length ranging from 2.67 mm to 3.68 mm.

Coloration: Head and clypeus dark brown; mouthparts, including palpus pale yellow. Antennae with scape, pedicel and flagellomeres 1- 4 pale yellow; remaining flagellomeres brown. Pleural sclerites brown. Scutum yellow with narrow longitudinal brown band dorsally. Scutellum brown. Mediotergite yellow laterally, brown posteriorly. Halteres whitish. Coxa whitish. Abdomen dark to light brown; hind margin of segment 1 – 5 with yellow markings extending laterally, contrast between yellow and brown varies between individuals (Fig. 3A). Tergite 7 light yellow, with lateral brown spot. Gonocoxites 8 brown. Cerci yellow.

Terminalia (Fig. 4A): Tergite 8, bare and elongated, gradually more membranous anteriorly. Tergite 9 and 10 reduced. Cerci two-segmented, first segment elongated, covered with small setae and with several long setae posteriorly; second segment small and rounded, covered with numerous small and several longer setae. Sternite 10 well developed and sclerotized, with minute trichia. Gonapophyses 9 fused to ventral side of sternite 10. Gonapophyses 9 well-developed and membranous, with several long setae apically. Gonocoxites 8 well developed, with several distinct setae apically, one particularly long. Sternite 8 extend into membranous labia.

Comments: The female genitalia in *A. zaitzevi* differ most distinctly from the other three studied species in the elongated first segment of the cerci, a clear row of setae at the dorsal posterior rim of gonocoxites 8, elongated segment 8 and Gonapophyses 9 with very long apex.

Allodia lugens Wiedemann, 1817

(Figs 3B, 3C, 4B)

Size: Total length ranging from 3.60 mm to 4.60 mm. Wing length ranging from 2.90 mm to 3.68 mm.

Coloration: Head and clypeus brown; mouthparts, including palpus yellow. Antennae with scape, pedicel and half of first flagellomere pale yellow; remaining flagellomeres brown. Pleural sclerites brown. Scutum brown, with narrow yellow lateral parts. Scutellum brown. Mediotergite brown. Halteres whitish. Coxa whitish. Abdomen dark brown, sometimes with yellow ventral markings on tergites 2 to 4, in clear contrast to dark brown areas (Figs 3B, 3C). Sternite 6 gradually yellow towards posterior border. Terminalia yellow.

Terminalia (Fig. 4B): Tergite 8 unmodified and bare. Tergites 9 and 10 reduced. Cerci two-segmented, first segment with distinct lump dorsally, covered with small setae and with several long setae dorsally; second segment small and oval-shaped, with some distinct setae, about two times as long as wide. Sternite 10 well-developed and scleritorized, with minute trichia. Gonapophyses 9 well-developed and fused with ventral part of sternite 10. Gonapophyses 9 membranous and triangular at apex. Gonocoxites 8 well-developed, with several distinct setae apically, one setae especially long. Sternite 8 extends into broad membranous labia.

Comments: The female genitalia of *A. lugens* differ most distinctly from the other three studied species in the lumped first segment, and in the shape of second segment of the cerci. The area representing sternite 10 is narrower and the labia broader.

Allodia pyxidiiformis Zaitzev, 1983

(Figs 3D, 5A)

Size: Total length ranging from 3.38 mm to 3.78 mm. Wing length ranging from 2.38 to 3.19 mm.

Coloration: Head and clypeus brown; mouthparts, including palpus, pale yellow. Antennae with scape, pedicel and half of first flagellomere pale yellow; remaining flagellomeres brown. Pleural sclerites brown. Scutum yellow with narrow longitudinal brown band dorsally. Scutellum brown. Mediotergite brown. Halteres whitish. Coxa whitish. Abdomen with segment 1-5 dark to light brown, with clear yellow lateroventral triangular markings (Fig. 3D). Tergite 6 dark brown and terminalia yellow.

Terminalia (Fig. 5A): Tergite 8 unmodified and devoid of setae. Tergites 9 and 10 reduced. Cerci two-segmented, first segment thick, with several short and long setae; second segment small and round, with several long setae. Sternite 10 well developed and fused with gonapophyses 9 ventrally. Gonapophyses 9 well-developed, with several setae at apex. Gonocoxites 8 well-developed and stout, with small setae at dorsal apex. Ventral side of gonocoxites 8 with several prominent setae posteriorly, one especially long, situated most apically. Labia membranous, covered with small tricia.

Comments: The female genitalia in *A. pyxidiiformis* differ most distinctly from the other three species in the short length of the terminalia. Moreover, the posteriorly truncated gonocoxites 8 and the shape of gonapophyses 9 differ from the other three species.

Allodia tuomikoskii Hackman, 1917

(Figs 3E, 5B)

Size: Total length ranging from 3.68 mm to 3.96 mm. Wing length ranging from 2.99 mm to 3.50 mm.

Coloration: Head and clypeus dark brown; mouthparts, including palpus pale yellow. Antennae with scape, pedicel and half of first flagellomere pale yellow; remaining flagellomeres brown. Pleural sclerites brown. Scutum brown dorsally, gradually becoming yellow laterally. Scutellum brown. Mediotergite brown. Halteres whitish. Coxa whitish. Abdominal segments 1-6 light to dark brown, mostly even color, but sometimes gradually light yellow vertically (Fig. 3E). Segment 6 with dark yellow posterior rim. Terminalia yellow.

The terminalia (Fig. 5B): Tergite 8 unmodified, devoid of setae. Tergites 9 and 10 reduced. Cerci one-segmented with many long and distinct setae dorsally and apically. Sternite 10 well-developed and sclerotized, with minute trichia. Sternite 10 fused with gonapophyses 9. Gonapophyses 9 membranous, apex with several small setae. Gonocoxites 8 well-developed, covered in small setae, with several pronounced setae apically, one especially long. Labia well-developed and located between gonocoxites 8. Labia membranous, covered with small trichia.

Comments: The female genitalia in *A. tuomikoskii* differ most distinctly from the other three studied species by the one-segmented cerci and the shape of the gonapophyses 9.

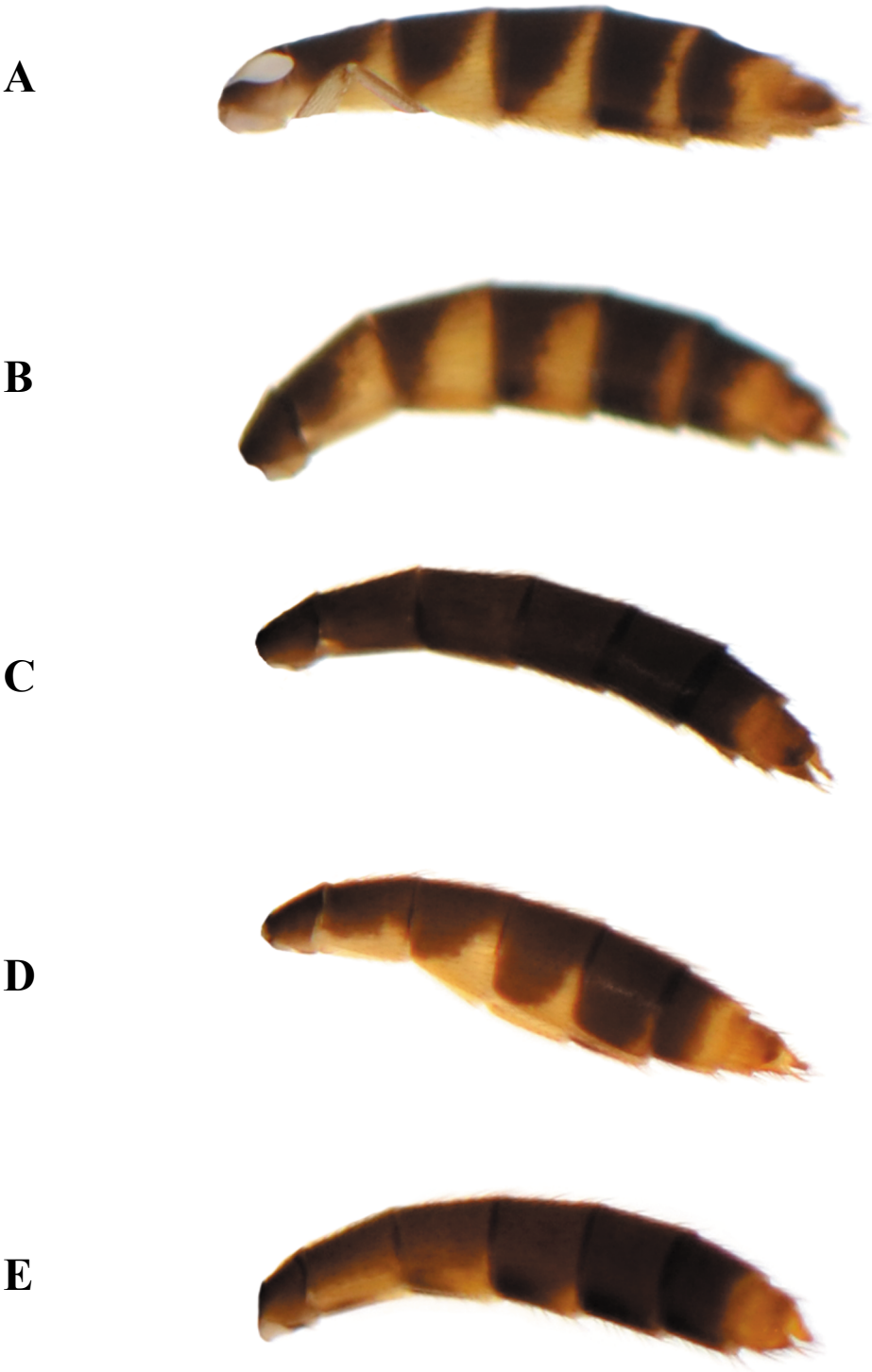

Figure 3: Females of *Allodia*, shape and color of abdomen. **–A**: *Allodia zaitzevi*, lateral view. **–B and C**: *Allodia lugens*, lateral view. **–D**: *Allodia pyxidiiformis*, lateral view. **–E**: *Allodia tuomikoskii*, lateral view.

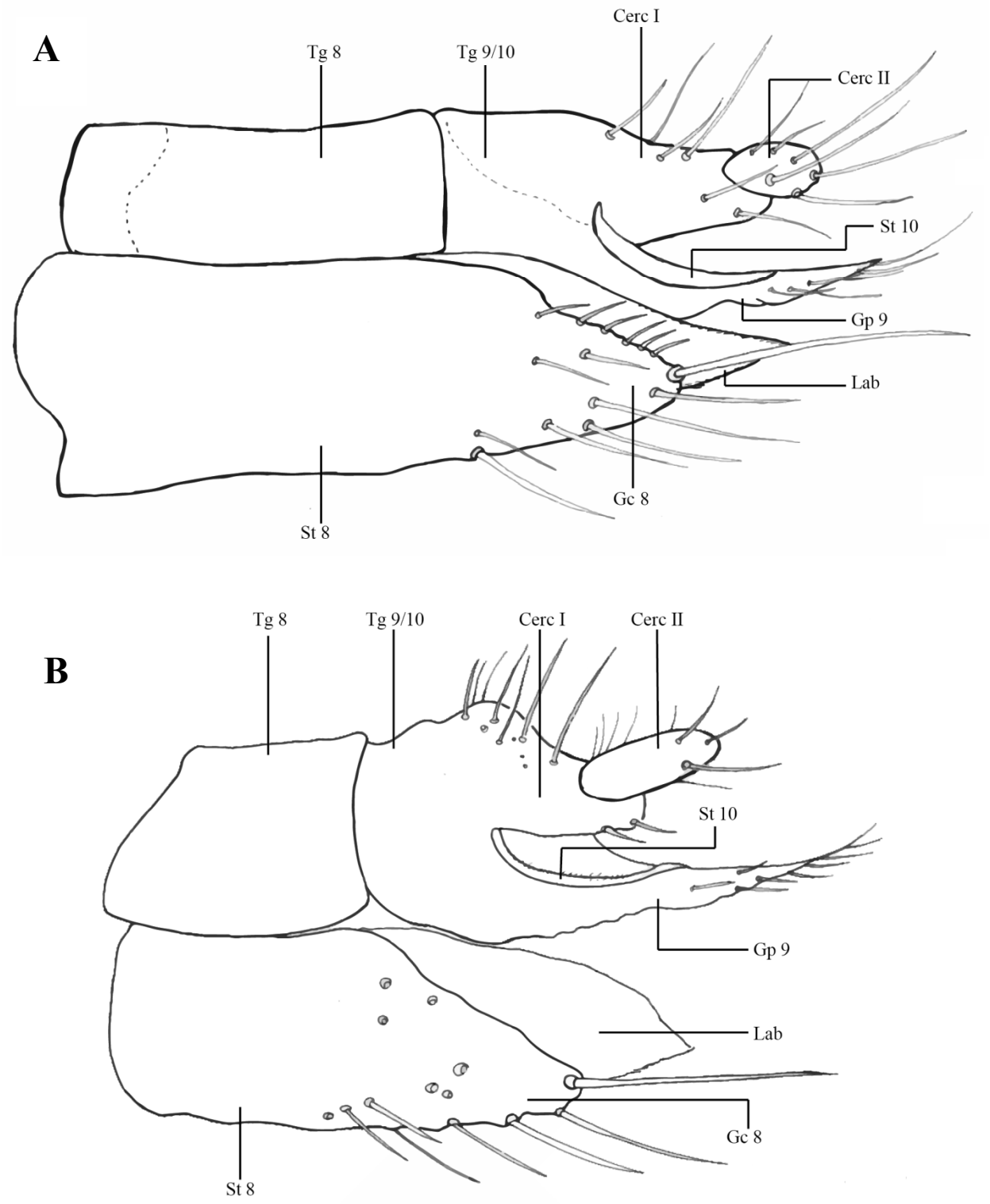

Figure 4: -A: Female terminalia of *Allodia zaitzevi*, lateral view. **–B:** Female terminalia of *Allodia lugens*.
Abbreviations: see figure 2.

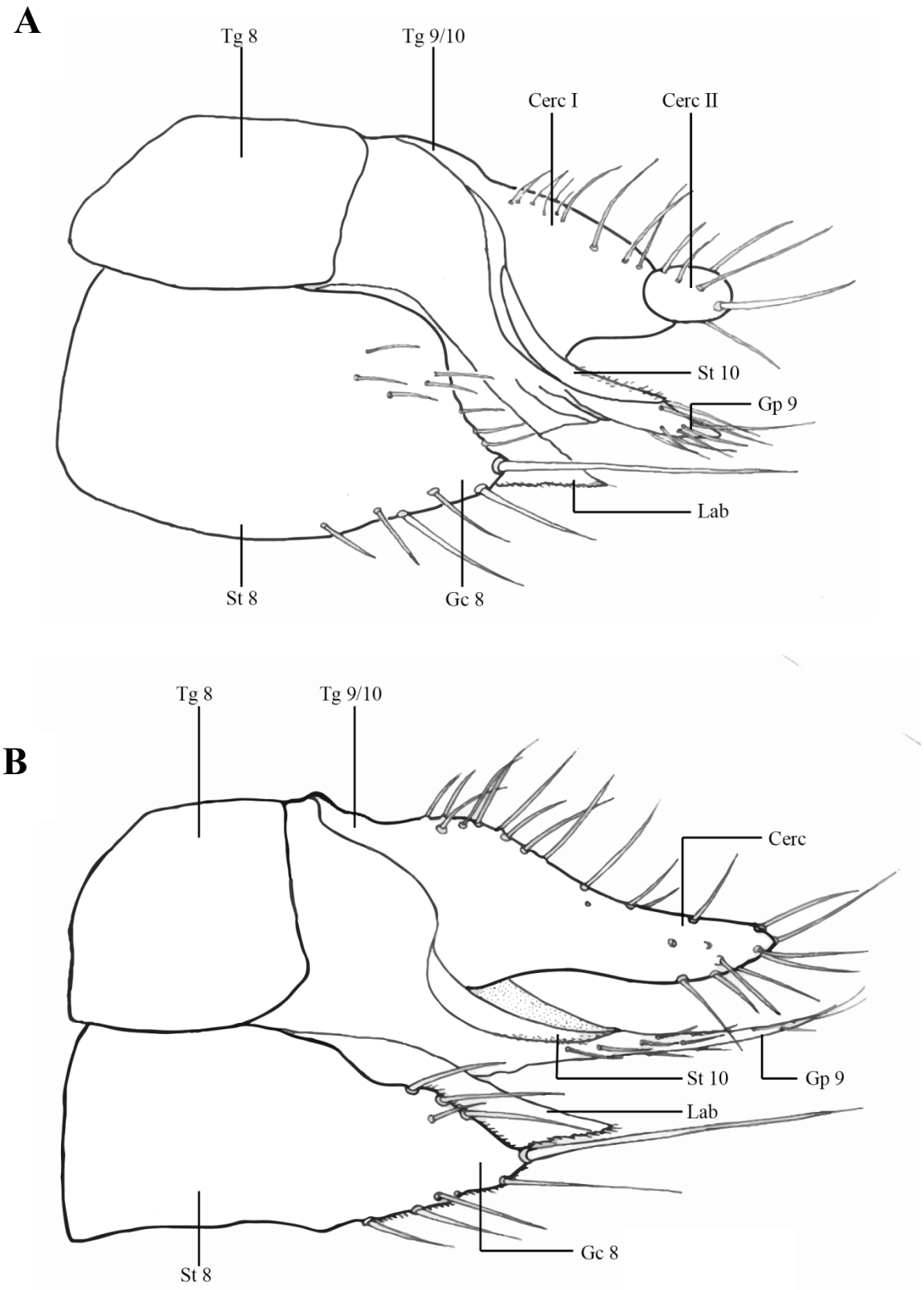

Figure 5: -A: Female terminalia of *Allodia pyxidiiformis*, lateral view. **–B:** Female terminalia of *Allodia tuomikoskii*, lateral view. **Abbreviations:** see figure 2.

Discussion

Kjærandsen (2006) reviewed the genus *Tarnania*, also belonging to the tribe Exechiini. The female genitalia in *Tarnania* have a very similar outline to that found in *Allodia*. A notable difference between the two genera is the presence of a well-developed tergite 9 in *Tarnania*, which is reduced in the studied species of *Allodia*. One of the females described by Kjærandsen, *Tarnania tarnani* Dziedzicki, 1910, also has a reduced tergite 9 and is very similar to the *Allodia* females here described. According to Kjærandsen, sternite 10 and gonapophyses 9 are fused in *Tarnania*. This interpretation is in accordance with the observations made in this study, although in *Allodia* it is possible to clearly separate the two structures. Kjærandsen have interpreted the structure termed labia in the present study as the gonapophyses 8 in *Tarnania*.

Females of *Allodia* are difficult to separate, and the present study shows that the four studied species can best be separated on structures of the female genitalia. This finding is in accordance with studies of the males of *Allodia* (Kurina 1997; Zaitzev 2003). As the coloration of both head and thorax is almost identical in the studied species, these characters can not be considered as appropriate characters to distinguishing between the species. The coloration of the abdomen varies between dark brown and yellow and there is little consensus within each species. This variation is particularly well expressed in *A. lugens* (see Figs 3B, 3C). Generally *A. tuomikoskii* has a more even color on the abdominal sclerites, and is slightly paler ventrally. Both *A. pyxidiiformis* and *A. zaitzevi* have clear lateroventral pale areas, but in *A. zaitzevi* the pale area extends much more dorsally.

It is important to note that all the material studied originate from the same locality, hence the intraspecific variation can not be ascribed local adaptation or variation. As seen in the association of females through DNA barcoding, 16 morphological groups turned out to represent only 7 species. This clearly illustrates the high variation within each species and demonstrates the usefulness of using DNA barcoding to associate the sexes.

Conclusive remarks

The association of gender by the use of DNA barcoding gave good results. The females from the genus *Allodia* clustered together with the already identified males in the Neighbor-joining analysis, with high bootstrap support. Four of the identified females were described, and the genitalia turned out to hold the most trustworthy morphological characters for separating the studied species, this probably also holds for other species within the genus. The differences are vague, but certainly serve to separate between closely related species.

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Appendix- Overview of terminology and synonyms

Table I: Terminology and synonyms, female terminalia of Trichoceridae

Present study	Saether (1977)	Dahl (1980)	McAlpine (1981)	Dahl and Krzeminska (1997)
Tergite 8	Tergite 8	Tergite 8	Tergite 8	Tergite 8
Tergite 9	Tergite 9	Tergite 9	Tergite 9	Tergite 9
Tergite 10	Tergite 10	Tergite 9	Tergite 10	Tergite 9
Cerci	Cerci	Ovipositor (derived from tergite 9)	Cerci	Ovipositor
Sternite 8	Sternite 8	Sternite 8	Sternite 8	Sternite 8
Gonocoxites 8	Gonapophysis 8	Part of sternite 8	Part of tergite 8	Part of tergite 8
Gonapophysis 8		Hypogynial valves, derived from segment 9	Hypogynial valve	Hypovalvae, derived from the segment 9
Gonapophysis 9	Labia ?	Vaginal plate	Sternite 9	Subgenital plate
Notum	Notum		Part of sternite 9	Vaginal apodeme
Sternite 10	Postgenital plate	Vaginal plate	Sternite 10	Supravaginal plate

Table II: Terminology and synonyms, female terminalia of Ptychopteridae

Present study	Peus (1961)	Saether (1977)*	Rozkosny (1997)	Andersson (1997)
Tergite 8	Tergite 8	Tergite 8	Tergite 8	Tergite 8
Tergite 9/10	Tergite 9	Tergite 9	Tergite 9	Tergite 9
Cerci	Cerci, first half interpreted as tergite 10	Two-segmented cerci	Cerci, first half interpreted as sternite 10	Cerci
Sternite 8	Sternite 8	Sternite 8	Sternite 8	Subgenital plate/ sternite 8
Gonocoxites 8	Part of sternite 8	Gonapophysis 8	Part of sternite 8	Part of sternite 8
Gonapophysis 8				
Sternite 9	Vaginal apodeme		Hpogynial valve	Posterior vaginal apodeme
Gonocoxites 9				
Gonapophysis 9	Sternite 9		Hypogynial valve	Sternite 9
Notum				
Genital plate	Vaginal apodeme		Hypogynial valve	Anterior vaginal apodeme

*: Saether (1977) studied the species *Bittacomorpha clavipes*, Fabricius 1781, with a very different morphology to *Ptychoptera minuta*, which is studied here.

Table III: Terminology and synonyms, female terminalia of Tipulidae

Present study	**Rees and Ferris (1939)**	**Byers (1961)**	**Frommer (1963)**	**Saether (1977)**
Tergite 8	Tergite 8	Tergite 8	Tergite 8	Tergite 8
Tergite 9	Tergite 9	Tergite 9	Tergite 9	Tergite 9
Tregite 10	Tergite 10	Tergite 10	Tergite 10	Tergite 10
Cerci	Cerci	Cerci	Cerci	Cerci
Sternite 8	Sternite 8	Sternite 8 (with first valvifers)		Sternite 8
Gonocoxites 8	Coxopodite			
Gonapophysis 8	Gonapophysis 8	hypovalve	Extensions of sternite 8 (=hypovalvae)	Gonapophysis 8
Gonapophysis 9	Sternite 9	Fused valvulae (ninth sternum)	Sternite 9	Sternite 9
Gonocoxite 9				Gonocoxite 9 fused with sternite 9
Genital plate		Furca	Vaginal apodeme, furca	
Sternite 10	Sternite 10			
Labia				Labia
Hypoproct			Infra-anal plate, posible sternite 11	Postgenital plate