RESEARCH ARTICLE

The Waterston's evaporatorium of Ceraphronidae (Ceraphronoidea, Hymenoptera): A morphological barcode to a cryptic taxon

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Abstract

The Waterston's evaporatorium (=Waterston's organ), a cuticular modification surrounding the opening of an exocrine gland located on metasomal tergite 6, is characterized and examined for taxonomic significance within the parasitoid wasp family Ceraphronidae. Modification of the abdominal musculature and the dorsal vessel are also broadly discussed for the superfamily Ceraphronoidea, with a novel abdominal pulsatory organ for Apocrita being discovered and described for the first time. Cuticular modification of T6, due to the presence of the Waterston's evaporatorium, provides a character complex that allows for genus- and species-level delimitation in Ceraphronidae. The matching of males and females of a species using morphology, a long standing challenge for the group, is also resolved with this new character set. Phylogenetic analysis including 19 Waterston's evaporatorium related characters provides support for current generic groupings within the Ceraphronidae and elaborates on previously suggested synapomorphies. Potential function of the Waterston's organ and its effects on the dorsal vessel are discussed.

Keywords

Cryptic species, exocrine, morphological barcode, pulsatory organ

Introduction

Ceraphronoidea is a cosmopolitan group of parasitoid wasps consisting of two extant families: Ceraphronidae and Megaspilidae. Although being one of the most abundantly collected Hymenoptera (Martinez and Murgia 2001; Schmitt 2004), the group currently comprises only about 660 described species worldwide (Johnson 2004). However, current estimates propose a significantly larger species diversity, with an estimated 329 species still unknown in Canada alone (Bennett et al. 2019).

The phylogenetic placement of Ceraphronoidea within Apocrita remains unresolved, with current molecular studies indicating a position as sister to Ichneumonoidea (Peters et al. 2017) or as sister to Evaniidae, in a clade that is sister to Aculeata (Tang et al. 2019). A sister group relationship to Ichneumonoidea is supported by limited morphological data, including the presence of semitransparent patches on the abdominal sternites (Trietsch et al. 2017). Likewise, the presence of numerous plesiomorphic traits, including the presence of a median mesoscutal sulcus, well developed pterostigma, two apical spurs of the foretibia, musculated harpes in the male genitalia, and the presence of T10 among others (Gibson 1985; Mikó et al. 2013), suggest Ceraphronoidea might retain more plesiomorphic features than most other Apocrita.

Phylogenetic relationships within the superfamily are yet to be resolved (Mikó et al. 2018). This uncertainty is due, in part, to a lack of molecular studies within Ceraphronoidea and the great difficulty in morphologically separating genera. Nine of the sixteen known genera of Ceraphronidae, for example, are rare, monotypic taxa, defined primarily by autapomorphies (Masner and Dessart 1967; Dessart and Cancemi 1987). Species-level characterization and delimitation is relatively robust, compared to other Hymenoptera, due to the informative nature of the male genitalia (Dessart 1963a; Mikó et al. 2018; Ulmer et al. 2018). To study the male genitalia of minute specimens, often less than a millimeter in length, is challenging. Many species descriptions, therefore, were based on ambiguous female traits, such as flagellomere ratios (Szelényi 1940), which have been shown to be unreliable due to the pronounced allometry within the group possibly due to their small size or variance in host size. (Dessart 1963b; Mikó et al. 2013). Male genitalia has become increasingly accessible, however, with the application of new imaging methods, such as confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and micro-computer tomography (μ-CT) and the application of glycerol-based preservation of specimens (Mikó et al. 2016).

The development of species concepts based primarily on male specimens, however, makes it difficult for one to identify conspecific females (Dessart 1963b). The matching of males with females within Ceraphronoidea, and Ceraphronidae in particular, has been a long standing issue with over half (60%) of all species descriptions being based solely on females within *Aphanogmus* Thomson, 1858 (46:100) and *Ceraphron* Jurine, 1807 (125:190) combined. This problem is further exacerbated with the monotypic genera, many of which are based only on a single female and hypothesized to simply be highly derived forms of the larger genera (*Retasus ater* Dessart, 1984; for

example, likely being a female *Aphanogmus* with unusual morphology) (Dessart 1984). Likewise, subgenera may be valid genera but lack robust morphological diagnoses of both sexes.

The abdominal Waterston's evaporatorium (=Waterston's organ) (WE) is a cuticular specialization on the sixth metasomal tergite (T6) that is composed of a smooth, concave region which is covered by a reticulate, honeycomb-like structure of cells in many taxa (Fig. 1A–C). The structure is a synapomorphy of Ceraphronidae, and Dessart (1975e, 1992) speculated that it might have potential for generic and even species characterization. Several hypotheses regarding WE function have been proposed since its original discovery by Nees (1834), including mechanosensory (Brues 1902) and respiratory (Waterston 1923), while more recent work suggests that the structure is associated with glands (Ogloblin 1944; Mikó and Deans 2009). Although some species- and genus-level variation in the WE has been recorded (Dessart 1963a, 1965a, 1980e, 1991b), the structure has never been described in detail.

The formation of new structures or changes in size of preexisting structures can often lead to cascading shifts in surrounding morphology (Nijhout and Emlen 1998). Due to internal and external morphology developing largely independently from one another. Alteration in sclerite morphology can alter muscle attachment points which in turn affect the mechanical function of said muscle (Matsuda 1976). Within Hymenoptera, the evolution of the petiole provides numerous examples of shifting musculature morphology in response to restructuring of sclerites (Vilhelmsen et al. 2010). The development of a petiole also leads to internal space constraints leading to novel developmental paths of the dorsal vessel in higher hymenopterans (Willie 1958). Exocrine glands cause both an external modification of the sclerite they are present on as well as an internal space constraint from the glands presence within the body cavity. The dorsomedian position of the Waterston's evaporatorium with its relatively large exocrine glands likely serve as spatial constraints within the ceraphronid metasoma. These in turn may trigger alterations not only in the skeletomuscular system but also structurally influence the dorsally located abdominal circulatory system. Understanding the broader phenotypic alterations in response to the presence of a structure allows for a more thorough morphological and evolutionary analysis of a structure and the taxa as a whole.

In this study, we provide a detailed description of the Waterston's evaporatorium and adjacent structural abdominal modifications and explore their taxonomic utility for genus- and species-level classification of Ceraphronidae. The structure's species specificity and phenotypic consistency between sexes is also investigated in order to facilitate matching males and females.

Materials and methods

Ethanol-preserved and freshly collected and fixed specimens of the present study have been deposited in the Frost Entomological Collection (PSUC). Morphospecies were
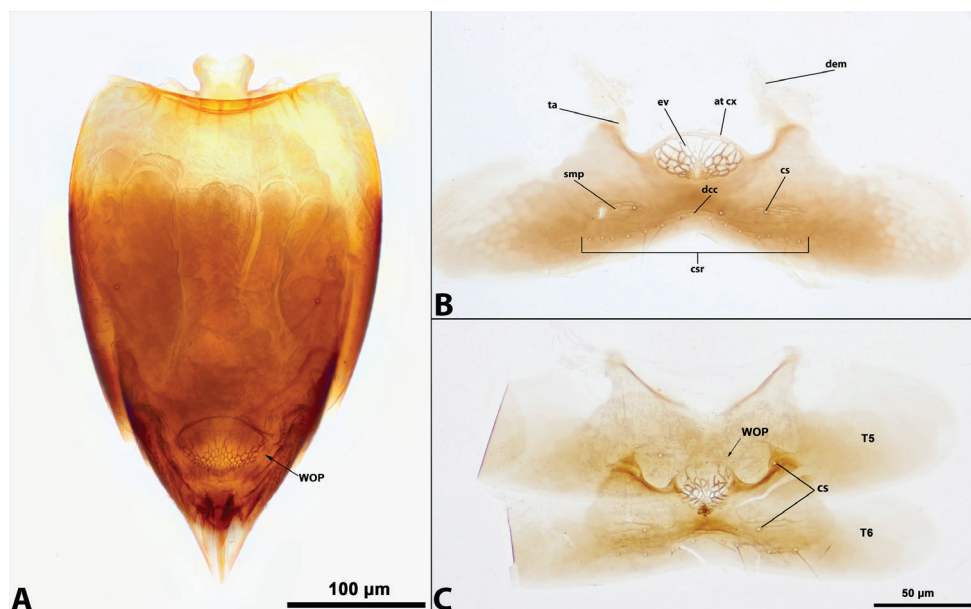
Figure 1. Morphological Terminology and overview of Waterston's evaporatorium (WE) and surrounding tergites in *Ceraphron* (brightfield) **A** metasoma in dorsal view, with WE visible on Mt6 **B** metasomal tergum 6 (T6) and Waterston's evaporatorium **C** metasomal tergum 5 and 6 ta = tergal apodeme ev = evaporatorium at cx = acrotergal calyx ite = intertergal extensor muscle sr ta = sclerotized ridge of tergal apodeme smp = submedial patches dcc = distal crenulate carina cs = campaniform sensilla csr = caudal setal row. Character abbreviations are provided in Appendix 1.

identified using male genitalia. Female specimens were taken from the same collecting event as males, when available. T6 was removed and mounted alongside the specimen.

Morphological terminology (Figs 1B, C; Appendix 1) was matched with concepts from the Hymenoptera Anatomy Ontology (Yoder et al. 2010). Terms referring to anatomical structures appear in bold and a URI table is provided (Suppl. material 2.) for terms in the HAO (http://portal.hymao.org/projects/32/public/ontology/). Abbreviations and figure references are given in parenthesis following the term. Abbreviations referring to muscles are italicized. New or modified terms are denoted with an asterisk (*). Skeletomuscular terminology for abdominal segments follows Snodgrass (1931) and Duncan (1939).

Dissections were performed under an Olympus SZX16 stereomicroscope with an Olympus SDF PL APO 1X PF objective (115×) and an Olympus SDF PL APO 2X PFC objective (230× magnification). Specimens were placed in glycerol, on concave microscope slides (Sail Brand CAT.NO.7103) and dissected with #2 pins and #5SA forceps. Brightfield imaging was performed using an Olympus DP71 digital camera mounted on an Olympus ZX41 compound microscope. All images were aligned and stacked (Pmax) using ZereneStacker v 1.04 (T201404082055). Scale bars were added in Adobe Photoshop 2020 (v 21.2.4).

Sample preparation for CLSM followed Mikó and Deans (2013). Abdomens of ethanol stored specimens were dissected and incubated at room temperature in
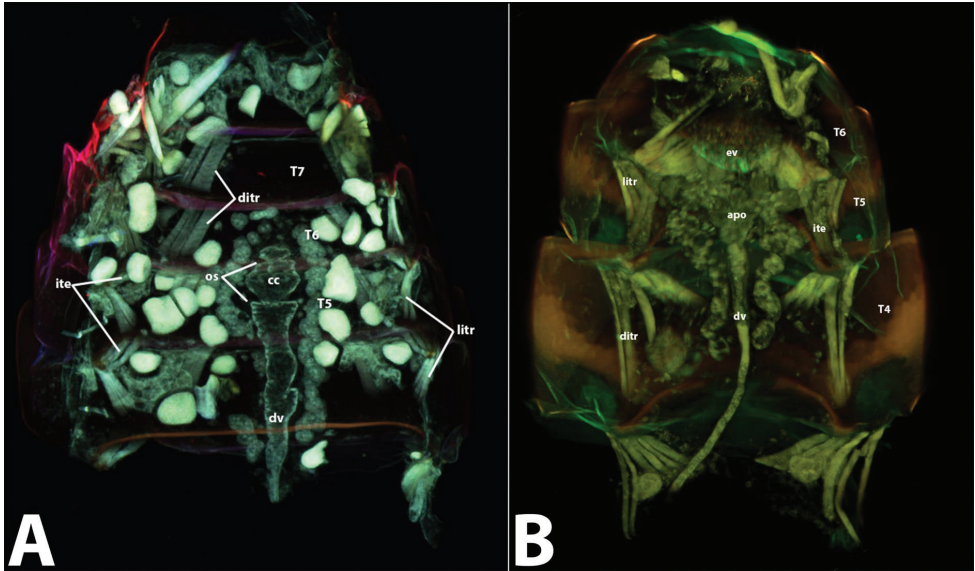
Figure 2. Skeletomusculature of Ceraphronoidea metasoma (CLSM) **A** dorsal view of metasoma of *Conostigmus* sp. showing termination of dorsal vessel into caudal chamber and tergal muscles **B** dorsal view of metasoma of *Ceraphron* sp. showing tergal muscles and termination of dorsal vessel into abdominal pulsatory organ anterior to Waterston's evaporatorium. Ditr = dorsal intertergal retractor muscles; ite = intertergal extensor muscles; litr = lateral intertergal retractor muscles; os = ostia; cc = caudal chamber; dv = dorsal vessel; apo = abdominal pulsatory organ; ev = evaporatorium.

35% hydrogen peroxide solution for 48 hours to bleach melanin-rich structures that may affect autofluorescence. Metasomal T6 of each specimen was then suspended in glycerol between two 0.15 mm thick, 24×50 mm coverslips and imaged with an Olympus FV10i confocal laser-scanning microscope (CLSM, Olympus Corporation of the Americas, Center Valley, PA) at the Microscopy and Cytometry Facility at the Huck Institutes of the Life Sciences at the Pennsylvania State University (Fig. 5C) and with a Nikon A1R-HD CLSM at the University of New Hampshire Instrumentation Center (Figs 2A, B, Figs 5A, B). With the Olympus FV10i, we used three excitation wavelengths, 405, 473, and 559 nm, and detected the autofluorescence using two channels with emission ranges of 490–590 and 570–670 nm (Fig. 2). On the Nikon A1R-HD, we used one excitation wavelength 487 nm laser with emission ranges defined using the A1-DUS spectral detector, 500–560 and 570–630 nm (Figs 3E and F, and 4–6). The resulting image sets were assigned pseudo-colors that reflected the fluorescence spectra. Volume-rendered micrographs and media files were created using FIJI (Schindelin et al. 2012) and Nikon NIS-Elements AR v. 5.02.01.

SEM micrographs were made with a Hitachi S-3200 Scanning Electron Microscope (wd = 23.5, av = 5 kV) at the Analytical Instrumentation Facility (AIF) of the North Carolina State University. Specimens were critical point dried and coated with palladium prior to imaging.
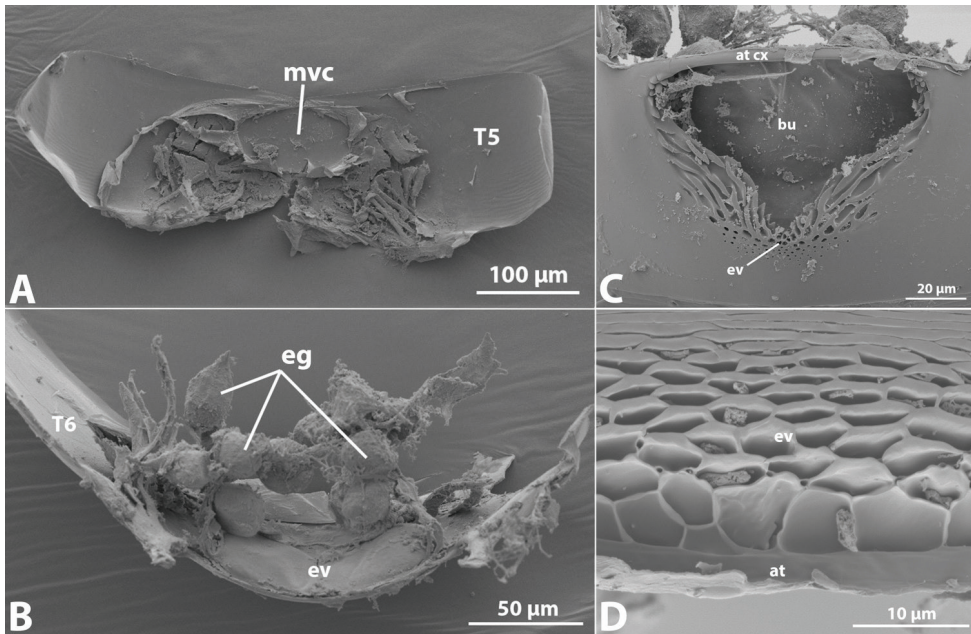
Figure 3. WE glands and sculpture of evaporatorium in Ceraphronidae (SEM) **A** ventral side T5 of *Aphanogmus* sp. showing median-ventral concavity corresponding to position of Waterston's organ on T6. **B** glandular subunits of the Waterston's organ extending into abdominal body cavity from ventral side of T6 and evaporatorium **C** evaporatorium and bulla of *Aphanogmus* **D** evaporatorium topography in *Ceraphron*, a reticulate matrix of empty cells. Mvc = median ventral concavity of T5 eg = evaporatorium glandules ev = evaporatorium at cx = acrotergal calyx bu = bulla; at = acrotergite.

Freshly collected specimens for serial block face scanning electron microscopy (SBFSEM) were dissected in 0.1M cacodylate buffer, fixed in 2.5% glutaraldehyde (in 0.1M cacodylate buffer), and then stained and embedded following Mikó et al. (2017). Specimens were then embedded in colloidal silver and trimmed with a Leica UCT ultramicrotome. Sectioning and imaging was conducted with a Zeiss SIGMA VP-FESEM with a Gatan 3View2 accessory at the Microscopy and Cytometry Facility at the Huck Institutes of the Life Sciences at the Pennsylvania State University. Images were aligned in imageJ (Version 2.0.0).

Characters were encoded within Mesquite Version 3.61 (build 927) then exported to TNT 1.5. The data matrix is available in Supplementary table 2. Cladistic analyses were performed with a traditional search, utilizing sub-tree pruning with all characters unordered. Collapsing rules were set to maximum length = 0. One thousand replications with 1,000 trees saved per replication were run, followed by branch breaking on optimal trees. A strict consensus was then run based on the resulting trees. Bootstrap support values were calculated from 10,000 pseudoreplications. Trees were post-processed in TreeGraph (version 2.15.0-887 beta).
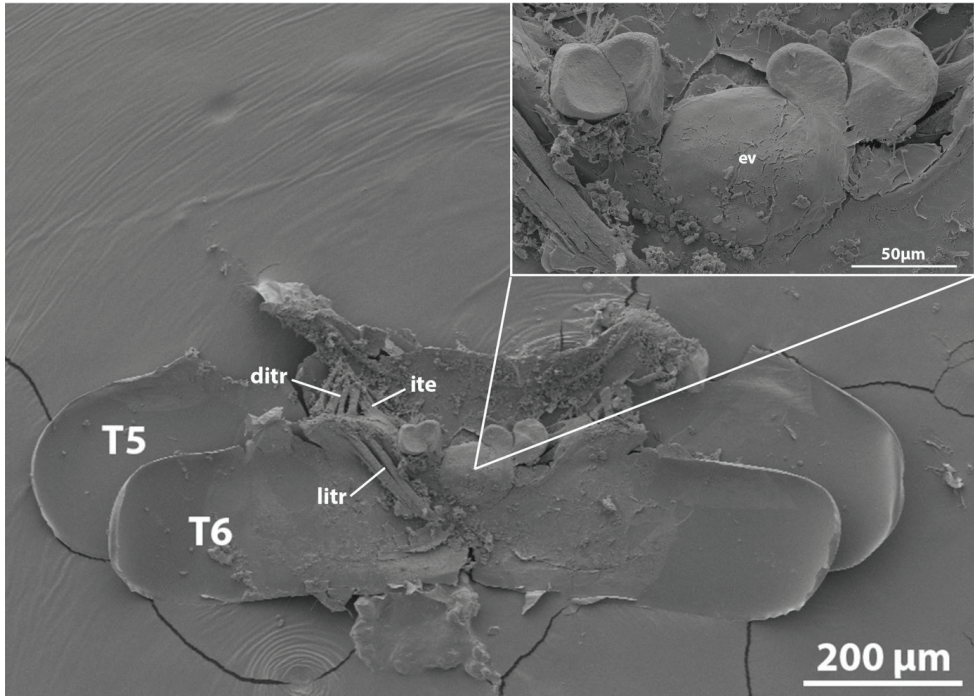
Figure 4. Tergite 5 and 6 of *Ceraphron* (SEM). The evaporatorium is visible ventrally as a plate corresponding to the bulla concavity or reticulate evaporatorium cell matrix. *Ditr* inserting lateral to *ite* on T6. *Litr* of T6 extending towards T7.

Results

Modification of the surrounding tergites and internal structures of Ceraphronidae in relation to the presence of the Waterston's evaporatorium are discussed in the following two subsections, these are provided as a comparative study relative to the sister group Megaspilidae.

T5 and T6 of Ceraphronoidea

The **Waterston's evaporatorium*** (**WE**) = (Waterston's organ) (Fig. 3B) is a cuticular specialization corresponding to a putative exocrine gland orifice present on the **acrotergite of metasomal tergite 6** (at: Fig. 3D). We use the name Waterston's evaporatorium to refer to the cuticular modification following Mikó et. al. (2009) due to the cuticular structure not falling within the definition of an organ as a multi-tissue structure.

In Megaspilidae, the abdominal tergites retain the plesiomorphic configuration; connected to each other by three muscles, the dorsal intertergal retractor muscle (***ditr***) (muscle 155, in T6; serially homologous with 133 in T4 and 144 in T5), which arises at the antecostal ridge of T5 and inserts medially on the antecostal ridge of T6. The lateral intertergal retractor muscle (***litr***) (muscle 156) (serially homologous with muscles
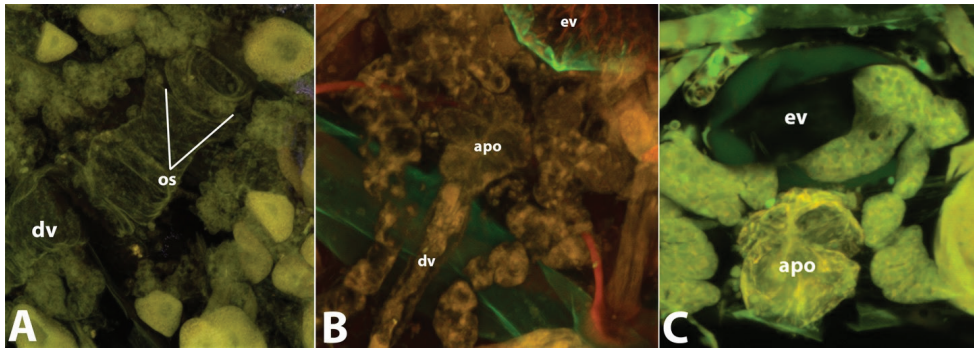
Figure 5. Dorsal vessel termination in Ceraphronoidea (CLSM) **A** dorsal vessel termination of *Conostigmus* sp. **B** dorsal vessel and abdominal pulsatory organ of *Ceraphron* sp. **C** abdominal pulsatory organ and evaporatorium in *Aphanogmus* sp.

134 [T4] and 145 [T5]) arises at the antecostal ridge of T6 diverging posteriorly from muscle 155 and inserts on the lateral edge of the **tergal apodeme** (**ta**) at the antecostal ridge of T6. The intertergal extensor muscle (***ite***) (muscle 157) (serially homologous with 135 [T4] and 146 [T5]) arises from the posterior margin of T5 and inserts apically on the tergal apodeme of T6. The antecosta does not bear any exocrine glands medially (Fig. 2A).

In Ceraphronidae, due to the presence of the WE, the tergal apodemes shift submedially on T6 and *ditr* is lateral to *ite* and the tergal apodeme (Figs 2B, 4). The **medioventral concavity*** (**mvc**) of T5 is a cuticular concavity on the ventral side of T5 corresponding to the WE of T6 which lies ventrally to the mvc when the *ite* muscles are relaxed. (Fig.3A) and bears campaniform sensilla at the level of the lateral tergal apodemes of the sixth tergite (T5) (Fig.1C). The T5 is not modified in Megaspilidae.

Internal anatomy

The exocrine glands corresponding to the WE are composed of Type III gland cells clustered ventrally to the WE. The glands attach via ductules connected with each glandular subunit which merge into larger ducts attached lateroventrally to the WE; the ducts terminate in the bulla or external reticulate cells of the WE. The glands themselves extend substantially into the abdomen (50–75 µm) (Fig. 3B).

In Megaspilidae, the dorsal vessel consists of a termination of the aorta into an enlarged globular caudal chamber ventral to T6, the caudal chamber contains an anterior and posterior pairing of ostia (os: Figs 2A, 5A). In Ceraphronidae, the dorsal vessel consists of a thin, weakly musculated aorta which abruptly terminates ventral to T6 and anterior to the WE into a large pulsatile organ consisting of 4–6 lobes divided by septa and musculated via broad alary muscles (Figs 2B, 5B, C).
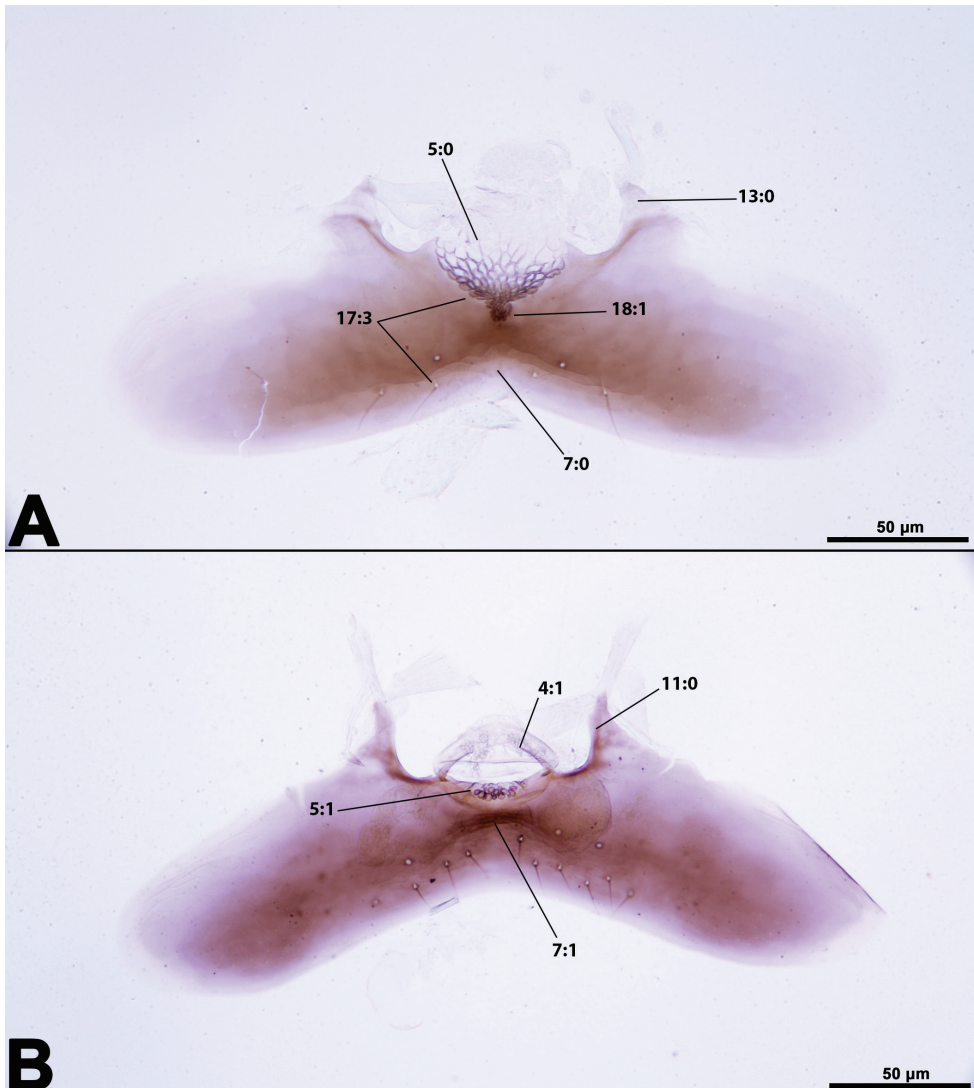
Figure 6. Principle differences between Waterston's evaporatorium in the *Ceraphron* Group and *Aphanogmus* Group (brightfield) **A** *Ceraphron* sp. **B** *Aphanogmus* sp.

Waterston's evaporatorium

The medial margins of the tergal apodemes are sclerotised in all members of the *Aphanogmus* group (Fig. 6B). In the *Ceraphron* group, the median margins of the apodemes are not sclerotized, but a sclerotized line traverses the apodeme basally (Fig. 6A). In *Elysoceraphron* and *Aphanogmus fasciipennis*, both sclerotizations (medial and basal) are present (Figs 7A, 8B; chr. 11). In *Trassedia* and *Masner*, the tergal apodemes are lo-

cated at the lateral edges of the tergum. In other ceraphronid taxa, the tergal apodemes originate at the lateral edges of the **evaporatorium** (**ev**) within the middle third of the tergite (chr.12). The evaporatorium consists exclusively of the medial reticulate cell structure while the term 'Waterston's evaporatorium' relates to the entire character complex. All Ceraphronidae *sensu stricto* have the tergal apodemes of T6 oriented submedially. The tergal apodemes converge or diverge proximomedially or are parallel to each other. Convergence is the only state found in the *Ceraphron* group (Fig.6A). In *Aphanogmus*, apodemes either diverge (Fig.8A) or are parallel (*A. fasciipennis* or *Synarsis*; Figs 8B, 7B; chr.13).

The WE is not reaching the lateral third of T6 in all Ceraphronidae aside from *Trassedia* in which it is present across the entire anterior margin of the T6 (chr. 2). The WE is transversely elongate and at least two times as wide as long in *Trassedia* and *Masner* and as long or longer than wide in Ceraphronidae *s.str.*

The **acrotergal calyx*** (**at cx**), the ridge arising medially from the acrotergite, forms the sclerotized distal edge of the **bulla** (**bul**), a smooth median concavity, present in all taxa within the *Aphanogmus* group (bul: Fig. 6B). Within the *Ceraphron* group, the bulla can be found only in *Pristomicrops*, *Cyoceraphron*, and *Pteroceraphron* (bul: Fig. 9). Within *Pteroceraphron* and *Pristomicrops*, the WE extends towards the acrotergal calyx (chr. 5:0), either up the lateral edges in *Pteroceraphron* or medially in *Cyoceraphron*. The cells of the evaporatorium extend beyond the acrotergite in all *Ceraphron* group taxa including those which contain an acrotergal calyx and a bulla. In these taxa the cells continue up the lateral edges of the bulla towards the anterior rim. Within the *Aphanogmus* group, the evaporatorium barely extends beyond the lateral walls of the bulla in some genera, e.g., *Synarsis* and *Elysoceraprhon* (Fig.7A,B).

The **caudal setal row** (**csr**), which arise from the posterior ridge of the tergite, is present in all ceraphronids with the number of setae being highly variable among genera (from 2–30), and intraspecific variation often being a difference of just 1–2 setae. The **distal crenulate carina*** (**dcc**)**,** on T6, a carina which arises dorsally to or upon the caudal setal row, is absent in *Ceraphron* (*Pristomicrops*), *Pteroceraphron*, *Gnathoceraphron*, and the examined specimens of *A. fumipennis* and *A. fasciipennis*. Dcc is present in all other genera. In the *Ceraphron* group, the distal crenulate carina always lies upon the caudal setal row when present (Fig. 6A). The position of the distal crenulate carina in many *Aphanogmus* group genera arises dorsally of the caudal setal row (Fig. 6B; chr.7).

The **submedian patches*** (**smp**) on T6 are located anterolaterally to the WE when present (smp: Fig.1B) (chr.8). The shape of the submedian patches is variable at the species level (chr.9). These patches have the campaniform sensilla within them (Fig. 10A). Submedian patches are only present in the *Ceraphron* group. In *Ceraphron* (*Eulagynodes*) sp. (Fig. 10B), the submedial patches are large and apparently continuous medially; they also connect to the distal crenulate carina which may obfuscate the carina. This is the character state determined for *Trassedia* and *Masner*; in both genera, cuticular sculpturing extends from the posterior edge of the evaporatorium to the distal tergal edge.
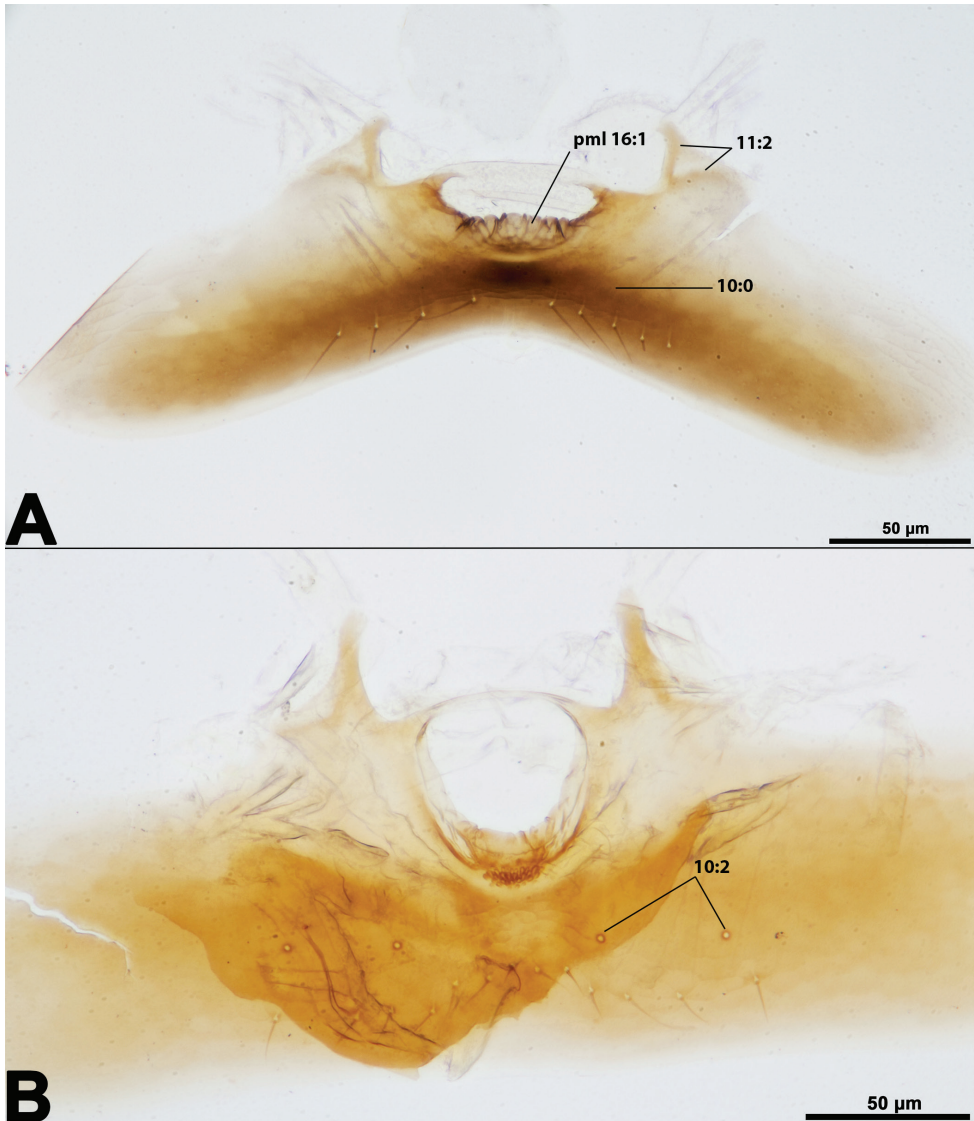
Figure 7. Variation of campaniform sensilla within the *Aphanogmus* Group (brightfield) **A** *Elysoceraphron hungaricus* **B** *Synarsis* sp. Presence of a complete proximomedial lamella (pml) (character 16) in *E. hungaricus* and partial pml in *Synarsis*. *Elysoceraphron* contains both inner marginal sclerotization and transverse sclerotization of the tergal apodeme. Both abnormal states of campaniform sensilla (character 10) are shown, *Synarsis* having two sets of cs, whereas they are entirely absent in *Elysoceraphron*.

A pair of **campaniform sensilla** are present laterally on both T6 and T5 in all Ceraphronidae except *Elysoceraphron* (Fig.7A). *Synarsis* have two sets of campaniform sensilla present on T6 (Fig.7B) (chr.10).
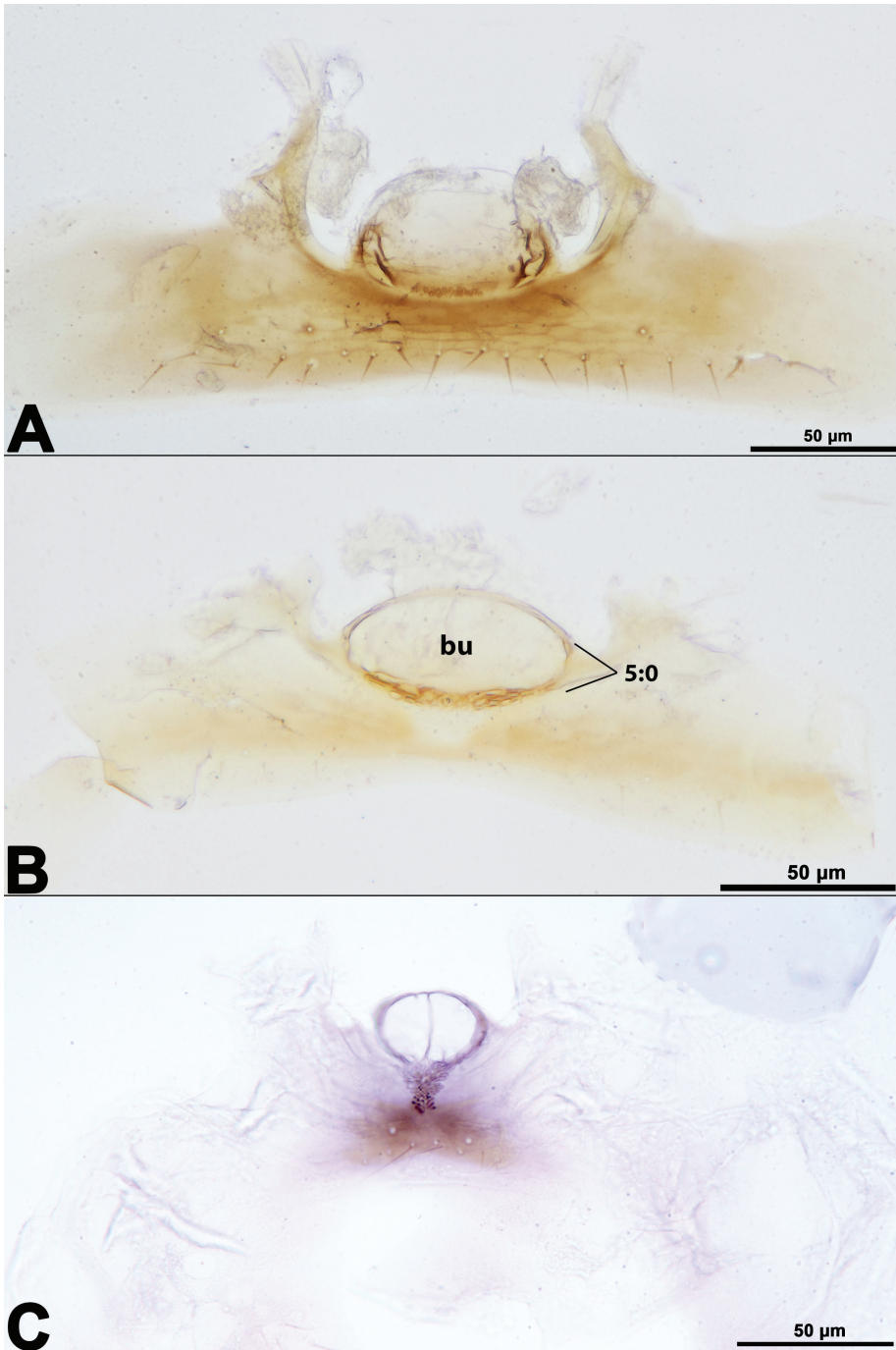
Figure 8. Acrotergal calyx within genera of *Ceraphron* Group (brightfield) **A** *Ceraphron* (*Pristomicrops*) sp. **B** *Pteroceraphron mirabilipennis* **C** *Cyoceraphron striatopleuralis*. Genera within the *Ceraphron* group which have the acrotergal calyx and bulla (**bul**) well separated. The evaporatorium extends up the lateral walls of the bulla in *P. mirabilipennis* (chr. 5)

Medially, unsculptured regions of the evaporatorium are only seen in some females of *Trassedia*, which, in several species, have a sexually dimorphic WE (chr. 14). Lighter melanization of the WE medial cells is found within *Ceraphron* (*Allomicrops*) (Fig. 10A; chr.15). This is different from char.14 where the evaporatorium is medially bifurcated by unmodified cuticle.

The **proximomedial lamella of the Waterston's evaporatorium*** (**pml**) extends from the base of the evaporatorium. In *Elysoceraphron*, the lamella envelops all cells of the evaporatorium (Fig. 7A). In *Ceraphron* (*Eulagynodes*), the lamella only covers the basal cells of the evaporatorium (10B; chr.16).

The uniformly-sized cells of the evaporatorium are smaller than or equal to the sizes of the setal sockets in *Aphanogmus* (Figs 8A, B). In *Ceraphron*, the cells increase in size as they extend towards the antecostal ridge (Fig. 6A). In *Pteroceraphron mirabilipennis*, the uniformly sized cells are larger than the setal bases (Fig. 9B; chr.17). A basomedial constriction of the WE is seen in many *Ceraphron* with the basal cells reducing greatly in size as they terminate into a single point (Fig. 6A) (chr.18). In the case of *Ceraphron*-type WE, the cells are often a combination of all size ratios, these are scored as polymorphic.

Sexual dimorphism is only found in some species of *Trassedia* where the female evaporatorium is medially unsculptured (Char.19, Mikó et al. 2018).

Character list

1. Evaporatorium: 0, absent; 1, present.
2. Evaporatorium lateral extension: 0, evaporatorium not reaching lateral third of tergite; 1, evaporatorium reaching lateral third of tergite.
3. Evaporatorium shape: 0, transversely elongate, at least twice as wide as long; 1, as long or longer than wide.
4. Acrotergal calyx: 0, absent; 1, present.
5. Evaporatorium cell: 0, cells extend to anterior rim of T6; 1, cells extend only to the edge of the acrotergite.
6. Distal crenulate carina on T6: 0, absent; 1, present.
7. Distal crenulate carina on T6 position: 0, present on caudal setal row; 1, present dorsally to caudal setal row.
8. Submedian patches on T6: 0, absent; 1, present.
9. Submedian patch medially: 0, patches continous medially; 1, patches medially separated.
10. Campaniform sensilla on T6: 0, absent; 1, single pair present; 2, two pairs present.
11. Sclerotized ridge of the tergal apodeme: 0, present along inner margin; 1, present, traversing base; 2, both.
12. Tergal apodeme shifted medially: 0, absent; 1, present.
13. Tergal apodeme orientation: 0, converging distally; 1, parallel; 2, diverging distally
14. Evaporatorium median unsculptured region female: 0, absent; 1, present.

15. Median cells of evaporatorium melanization: 0, lightly melanized; 1, heavily melanized.

16. Proximomedian lamella of T6: 0, absent; 1, present.

17. Evaporatorium cell size related to setal bases: 0, smaller than setal base; 1, equal to setal base; 2, larger than setal base.

18. Evaporatorium basomedial constriction: 0, absent; 1, present.

19. Dimorphism (Only *Trassedia*): 0, absent; 1, present.

Results of cladistic analysis

Parsimony analysis resulted in 92 trees with a length of 136 steps (Fig. 11). The strict consensus tree retrieved the following relationships:

"*Ceraphron* group"

The *Ceraphron* group is paraphyletic to the *Aphanogmus* group (see below), and includes *Ceraphron sensu stricto*, as well as its included subgenera (*C.* (*Allomicrops*), *C.* (*Pristomicrops*), *C.* (*Eulagynodes*), *Homaloceraphron*, *Pteroceraphron*, *Cyoceraphron*, and *Ecitonetes*. The group is characterized by the absence of an inner marginal sclerotization of the tergal apodeme, with the apodeme being sclerotized transversely across the base and converging distally. The extension of the evaporatorium to the anterior rim of the tergite is also a character shared by members of the group including those genera that have an acrotergeral calyx wherein the evaporatorium extends along the edges of the formed bulla.

"*Aphanogmus* group"

The *Aphanogmus* group (*Aphanogmus sensu stricto*, *Synarsis*, *Elysoceraphron*, *Gnathoceraphron*) is supported by the presence of an inner marginal sclerotization of the tergal apodemes and their subsequent distal divergence or straightening. The presence of an acrotergal calyx and the evaporatorium not advancing beyond the acrotergite. This grouping is strongly supported as monophyletic with a bootstrap (BS) value of 89.

"*Trassedia* and *Masner*"

Trassedia is well supported (BS value: 97) and characterized by the presence of dimorphism (chr. 19) and a medially unsculptured region of the evaporatorium in females (chr. 14). *Masner* is present within a trichotomy also comprising *Trassedia* and the rest of Ceraphronidae *sensu stricto*. *Trassedia* and *Masner* are characterized by a lack of tergal apodeme modification as well as a laterally extended evaporatorium present directly along the acrotergite without any modification into a calyx. *Masner lubomirus* (Mikó & Deans, 2009) is only known from male specimens, so the presence of dimorphism is unknown for the genus.
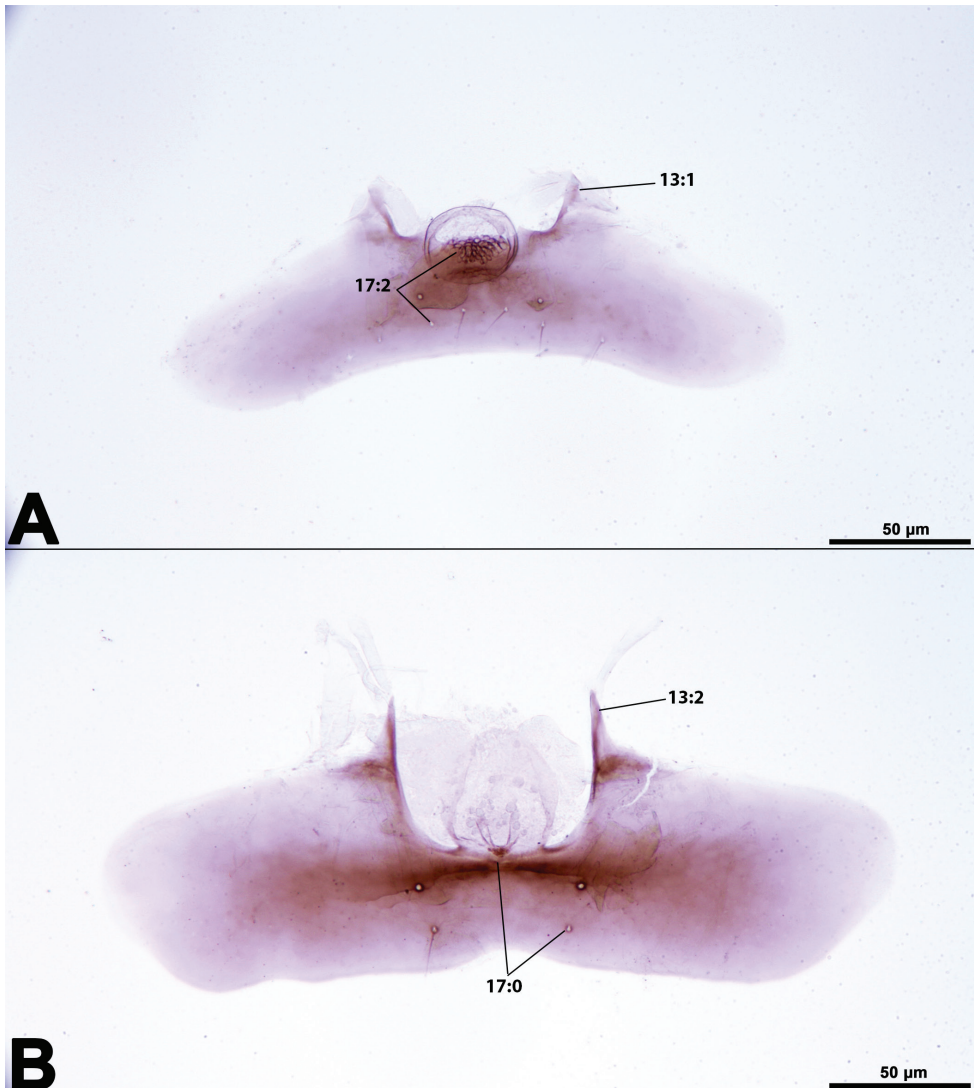
Figure 9. Variation of Waterston's evaporatorium between two major species complexes of *Aphanogmus* (brightfield) **A** *Aphanogmus fumipennis* **B** *Aphanogmus fasciipennis*. Character 13 = Tergal apodeme orientation. Character 17 = cell size in relation to setal base.

Taxonomic utility

Interspecific variation in the WE is focused primarily in the gestalt of the evaporatorium, with the relative size and sclerotized pattern of the reticulate network providing the clearest initial indicators of species variability (Fig. 12). Within *Ceraphron*, morphospecies based on male genitalia maintain the generic apomorphies (basomedial constriction, transverse sclerotization of the ta, smp), there is however, distinct
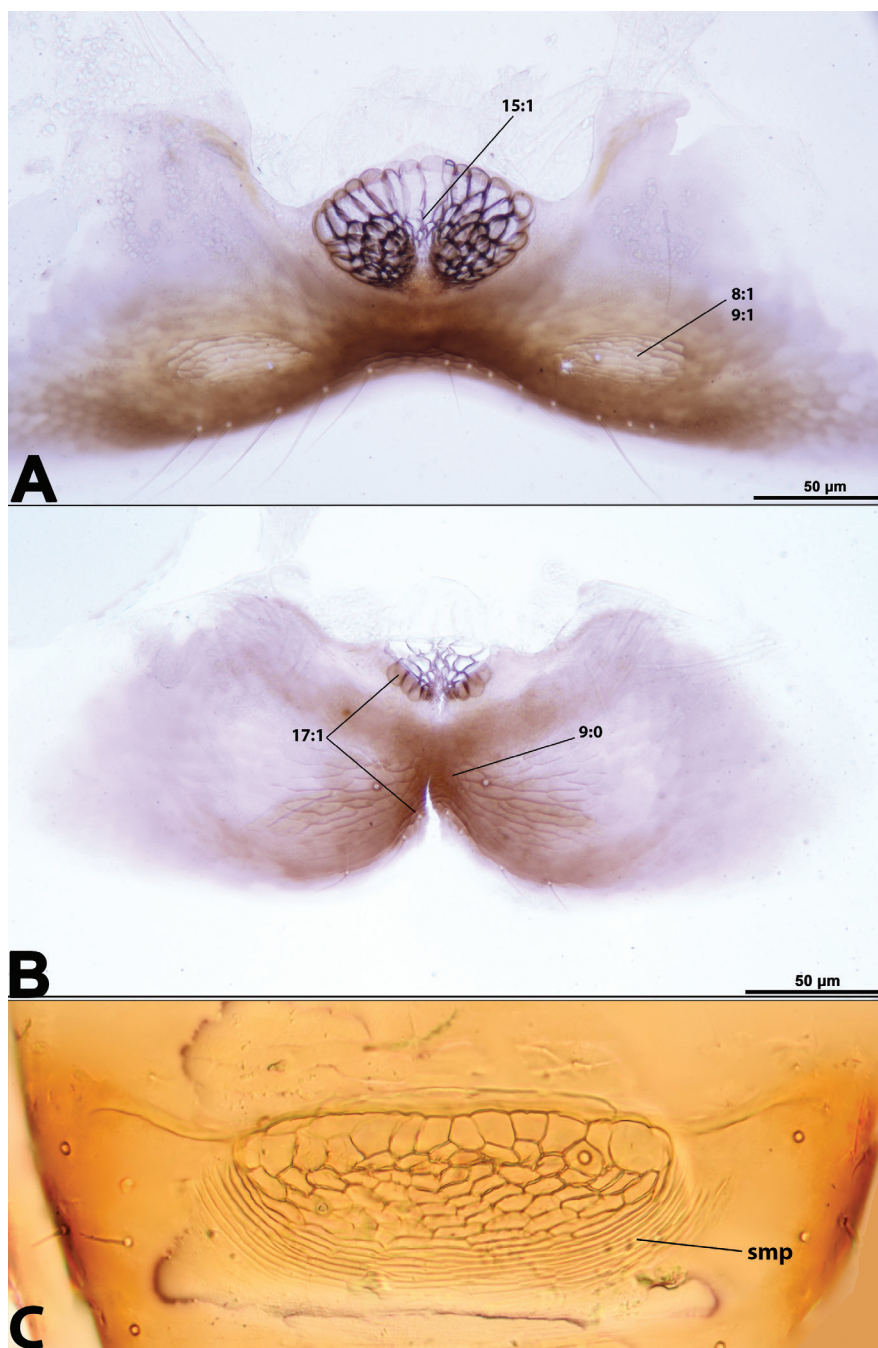
Figure 10. Variation in submedial patches within subgenera of *Ceraphron* (brightfield) **A** *Ceraphron* (*Allomicrops*) sp. **B** *Ceraphron Eulagynodes* sp. **C** *Masner lubomirus*. Characters states associated with the presence of smp (character 8). Lighter medial melanization of the evaporatorium found in some species of *Ceraphron Allomicrops* (chr. 15). The three states of character 9 can be seen, medially separated in *C. Allomicrops*; and medially continuous, in *C. Eulagynodes* and *M. lubomirus*.
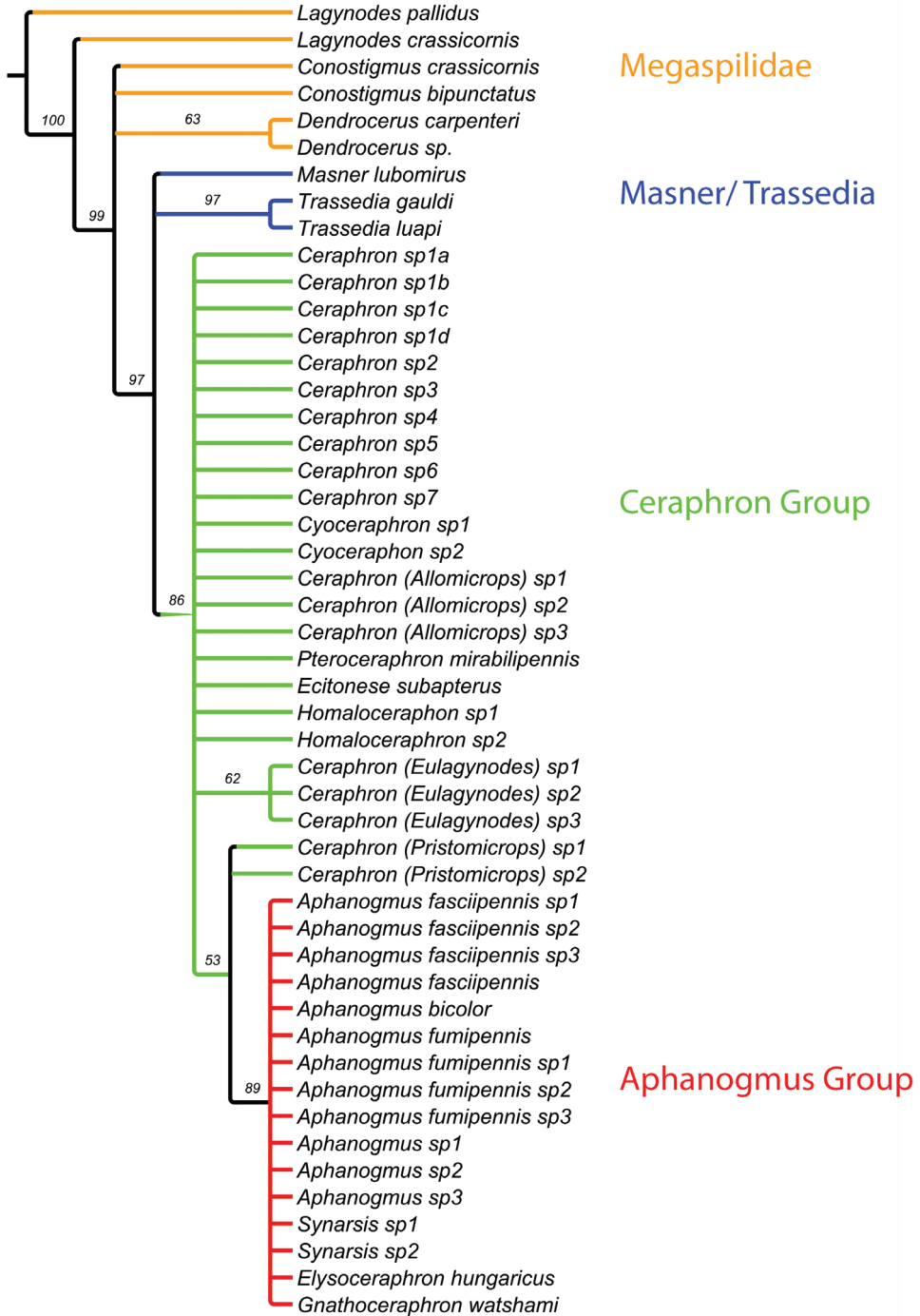
Figure 11. Phylogenetic relationships within Ceraphronoidea inferred by morphological characters from the Waterston's evaporatorium. Strict consensus tree with bootstrap support values higher than 50. Tree length = 136.

variation within these which as a combination of characters can be informative on the species level.

Within a single morphospecies the intraspecific variation results from a variation in the number of setae along the caudal margin, as well as the sclerotization of the evaporatorium with the darkness of the medial region being relatively variable (Fig. 13). Size variation in the tergite itself is the result of body size variability within a given species.

The structure is monomorphic between the sexes of a given species, with the intraspecific variation amongst males examined extending to the variation between conspecific males and females (Fig. 14). During the examination of males and females, it was noted that in many matched species the basomedial cells of the male tend to be strongly sclerotized relative to the corresponding female. Whether this is related to a function or a form of dimorphism in the structure is presently unknown.

Discussion

Modification of T5 and T6 and the presence of anterior tergal glands

Abdominal cuticular modifications corresponding to abdominal exocrine glands have been described in numerous other hymenopteran taxa, including Braconidae (Buckingham and Sharkey 1989; Quicke 1990), Platygastroidea (*Xenomerus*, Mikó et al. 2010), and recently in one of the proposed sister taxa to Ceraphronoidea, Megalyridae (Mikó 2014). In the case of Braconidae, these structures have provided a useful character complex for both tribal and generic level classification (Buckingham and Sharkey 1989; Sharkey 1992). Emptying of these glands might be regulated by the telescopic movement of the tergites relative to each other (Mikó 2014) that is performed by the alternate contraction of the internal and external abdominal musculature.

Novel abdominal pulsatory organ in Apocrita

The dorsal vessel in Ceraphronidae terminates into an enlarged, multi-chambered accessory pulsatory organ that has never been reported in Apocrita (APO) (Fig. 5B, C). Within the sister family Megaspilidae, the dorsal vessel also terminates in a caudal chamber, however, it is not as large and is not divided into multiple chambers by septa. Pulsatory organs are usually developed to facilitate circulation in extremities like the insect appendages, antennae, wings and legs (Pass 2000).

In Ceraphronidae, where the WE and adjacent exocrine glands take up a considerable amount of internal space, the complex might represent a barrier between the anterior and posterior sections of the abdomen (Fig.3B). A larger and more heavily musculated pulsatile organ may be necessary in order to provide appropriate hemolymph flow around the obstructive glands to more posterior organs including the sometimes disproportionately large and complex genitalia (Dessart 1995; Mikó et al. 2013; Ulmer et al. 2018).
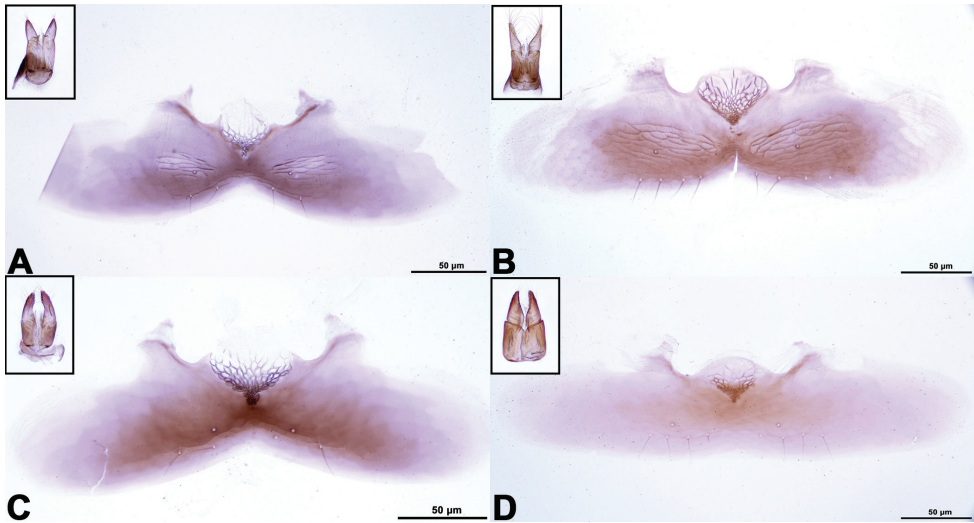
Figure 12. Species level variation of WE within morphospecies of *Ceraphron* (brightfield). Morphospecies based on male genitalia (inlays). Four distinct smp patterns and evaporatorium's.

The dorsal vessel is relatively short in both ceraphronoid families and potentially corresponds to the ability to telescope the entire abdomen into the syntergite. The presence of an enlarged caudal chamber of the dorsal vessel would provide circulation when the abdomen is fully extended.

The formation of a caudal chamber at the end of the dorsal vessel is a common trait found within wingless Hexapoda groups (Diplura, Zygentoma, and Archaeognatha). Some Pterygota, such as Ephemeroptera and Plecoptera, have a "pear-shaped" termination to the dorsal vessel, which functions as an accessory pulsatile organ for the abdominal appendages, including the cerci (Gerebren and Pass 2000; Pass 2000; Pass et al. 2006). A unique modification of the dorsal vessel into an enlarged lobed pulsatile organ has been described within *Acheta domesticus* (Linnaeus, 1758) (Orthoptera: Gryllidae), which allows for the backflow of hemolymph through its long ovipositor (Hustert et al. 2014). Unfortunately the study did not examine males to determine if this structure is found within both sexes with a redundancy in males, as could potentially be the case for the WE in Ceraphronidae.

Variations in the path of the dorsal vessel through the mesosoma and petiole have been examined across Hymenoptera, showing a remarkable range of diversity given the highly conserved nature of the structure (Matus and Pass 1999; Hillyer and Pass 2020). Amongst Hymenoptera, Euglossini (Apidae) have a unique shortening of the dorsal vessel into a caudal, enlarged chamber (Willie 1958). Functionally, the caudal chamber adaptations found within Ceraphronoidea and Euglossini are likely analogous to the enlarged "abdominal heart" and thin aorta found within several Hemipteran families, in which the heart is highly musculated via broad alary muscles and the aorta has little or no contraction power for circulation (Chiang et al. 1990; Hinks 1966; Pass pers.
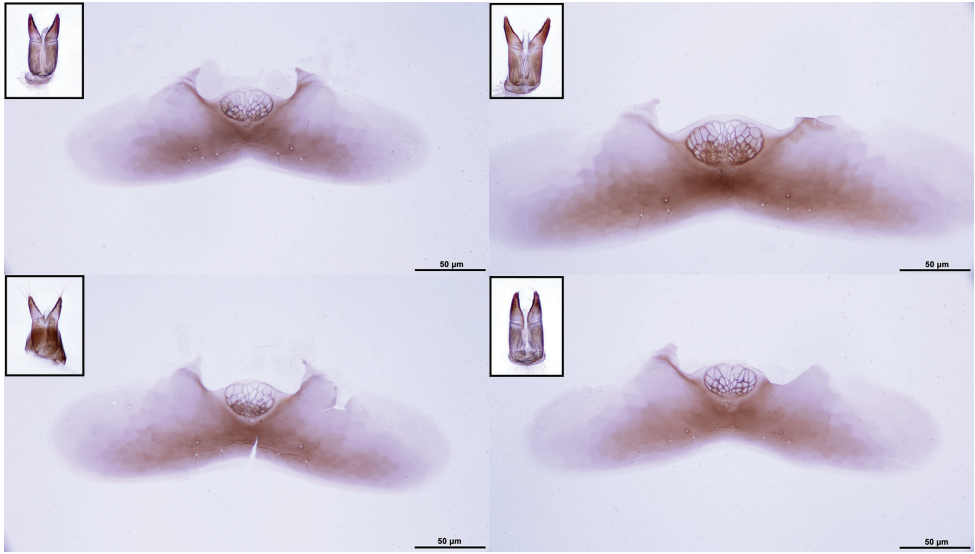
Figure 13. Intraspecific variation of WE within a single morphospecies of *Ceraphron* (brightfield). Morphospecies based on male genitalia (inlays).

comm.). We hypothesize that the presence of a caudal abdominal organ is not unique to Ceraphronoidea. Studies on the circulatory system in Hymenoptera are remarkably limited, especially for the minute parasitoid wasps (Quicke 1997) and future studies should explore the nature and phylogenetic significance of this complex anatomical system for Hymenoptera evolution.

Glandular function

The class III gland cells (Noirot and Quennedey 1974) along with the modification of the modified tergal apodemes strongly suggests an exocrine glandular function of the Waterston's evaporatorium (Mikó and Deans 2009). It is possible that these glands could be utilized in courtship rituals, as the glands are often monomorphic when present (Quicke 1997). Glands utilized in courtship allow for premating selection of compatible mates amongst cryptic species complexes or provide individual specificity in the case of lek mating to prevent repeated mating between two individuals (Assem 1994; Ayasse 2001). The tergal and sternal gland secretions of Opiinae (Braconidae: Ichneumonoidea) have been found to be species-specific in their chemical composition, with this specificity proposed as functioning in mating or courtship (Williams et al. 1988).

Another potential function is as a defensive gland against terrestrial predators or fungal parasites. Due to the diversity of hosts even within a single genus of Ceraphronidae (Peter and David 1990), the specialized adaptation of the structure to a specific habitat and its predatory fauna is unlikely, and its use as a deterrent to microbial infection is more probable if host-specificity is an assumed evolutionary driver. While
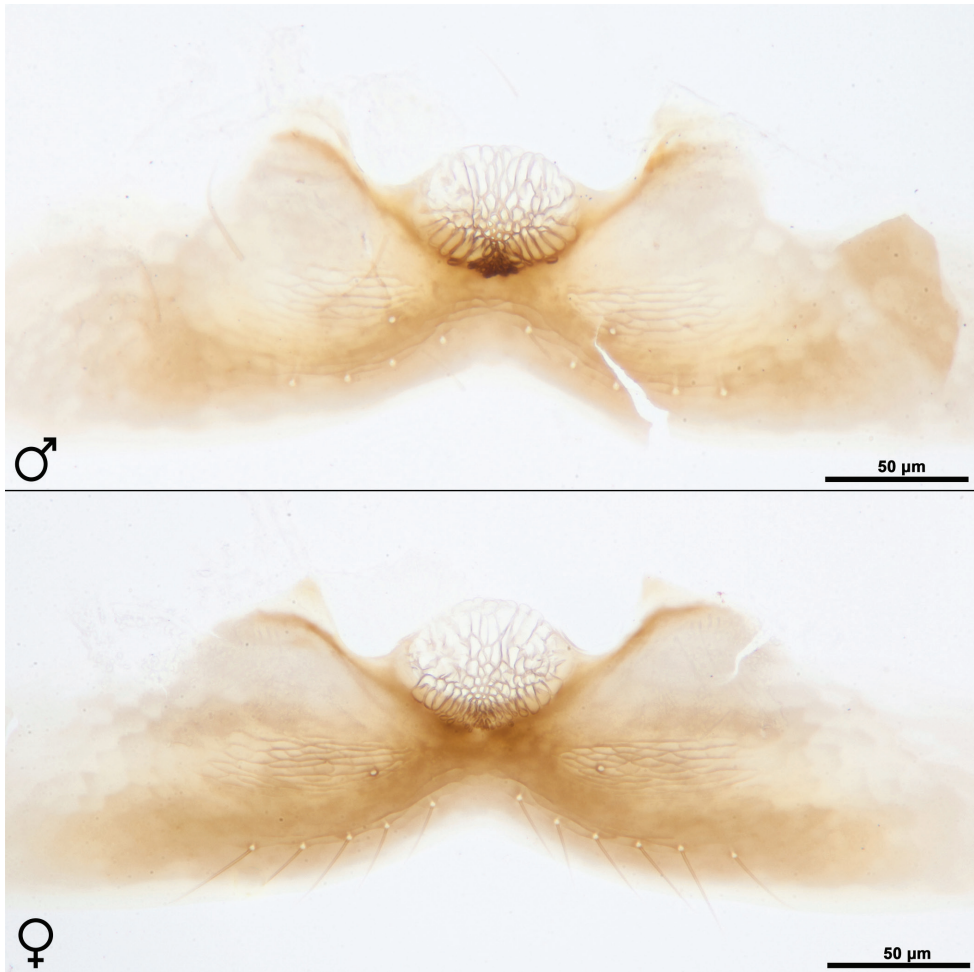
Figure 14. Morphological barcoding (brightfield). Matching male and female *Ceraphron* (*Allomicrops*) based on WE.

there are limited records of natural history, Ceraphronidae is often collected in pan traps or sweep netting which may indicate a ground-level or subterranean life history; this is in contrast to Megaspilidae, which is more commonly collected in flight intercept and Malaise traps (pers. obs.). Many arboreal ant genera have been found to have atrophied metapleural glands which may suggest a reason for the absence of the WE in Megaspilidae (Hölldobler et al. 1984). The putative defensive or antimicrobial function of the WE could be analogous to the metapleural glands of Formicidae. The metapleural gland excretion has been shown to be a weakly acidic compound that functions as an antimicrobial agent that is collected into a bulla structure and used in grooming (Beattie et al 1986; Fernández-Marín et al 2006). Further studies into the chemical makeup of the gland excretion of the Waterston's evaporatorium may further elucidate potential functions.

Phylogenetic implications

A limiting factor in resolving the generic and species level relationships within Ceraphronidae is the paucity of characters that are not sex specific. While family level separation is robust, based on ten two-state characters, one of which is the presence of a Waterston's evaporatorium, generic concepts lack the same degree of resolution (Mikó and Deans 2009). Dessart and Cancemi (1987) classified Ceraphronidae into "satellite" groups based upon compression of the mesosoma and male flagellomere shape. The WE character complex provides further evidence for this grouping (Fig. 11), particularly in those genera which are monotypic or only known from females.

The absence of an acrotergal calyx (ch.4:0) is implied to be the ground plan for the group in our analysis, lacking from the sister group Megaspilidae, *Masner* and *Trassedia* as well as from *Ceraphron*. Presence of an acrotergal calyx within *Pristomicrops*, *Homaloceraphron*, and *Pteroceraphron* in combination with the presence of evaporatorium cells extending along the lateral edges towards the anterior rim of T6 (ch.5:0) suggests a possible transformation series towards the presence of an acrotergal calyx and bulla found within all *Aphanogmus* and reduction of the evaporatorium cells to a small basomedial patch on the acrotergite.

Sclerotization of the inner margin of the tergal apodeme is also found to be plesiomorphic, (ch.11:0) being the state found in the Megaspilidae as well as in *Masner* and *Trassedia*, which are sister to Ceraphronidae s.s. Having the sclerotization traverse the base of the apodeme is a putative apomorphy for the *Ceraphron* group, with a reversal in *Aphanogmus*. Whether the state found in *Ceraphron* is associated with the tergal apodemes converging distally (ch.13:0) is uncertain. This transverse sclerotization however is not seen on the preceding tergal apodemes on T5, which in all ceraphronoids appear unmodified, being sclerotized along the inner margins and diverging distally (Fig.1C). In *Elysoceraphron hungaricus* (Szelényi 1936) and the *A. fasciipennis* complex, both inner margin sclerotization and sclerotization traversing the base of the apodeme are present, with the apodemes diverging distally or straight as in all other *Aphanogmus*-group genera.

Morphological barcoding

It has been notoriously difficult to obtain molecular barcodes for Ceraphronoidea (Ratnasingham and Hebert 2007; Bennett 2019). This has manifested itself taxonomically in a general inability to obtain species or even genus matches between specimens of opposite sex. However the taxonomic utility of barcoding cannot be overstated. While molecular barcoding is the standard medium within biology, the ultimate purpose of this tool is to develop discrete signatures efficiently which can be assigned to a taxon in order to differentiate it. Waterston's evaporatorium might offer an alternative to molecular barcoding for Ceraphronoidea, until the molecular impediment has been resolved within the group.

As one reaches lower taxonomic levels, characters naturally become more subtle and limited, as evolutionary distance is shortened. This is a problem which plagues both morphological and molecular taxonomy (Song et al 2008). In order to most effectively utilize the WE character complex to species level, inter- and intraspecific variation must be quantified (Fig. 12; 13). This issue has been partly resolved in molecular taxonomy through the use of large reference libraries. Pattern recognition software such as I3S, has proven capable of differentiating species and even single specimens (Sannolo et al 2016) with increasing accuracy as a reference library grows. While the reticulate cell structure of the evaporatorium is largely conserved within a given taxon, intraspecific variation is an inescapable aspect of evolution which may be accounted for through this method of reference library generation. Future studies should take care to preserve and image the WE in order to better understand its utility.

While male genital characters remain a far more robust system for diagnosing species, they are obviously limited by the sex of the specimen. Currently, the intraspecific characterization of the WE is the only way to confidently match sexes within Ceraphronoidea (Fig. 14). The WE would thus provide the ability to associate the sexes in these groups, which can then be diagnosed with male genitalia. We hope that use of this character set will only further bolster its application and utility within the family as more WE are described and defined.

Acknowledgements

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Appendix I

List of abbreviations for structures:

WE = Waterson's evaporatorium = (Waterston's organ)
ta = tergal apodeme
ev = evaporatorium
at = acrotergite
at cx = acrotergal calyx
bu = bulla
sr ta = sclerotized ridge of tergal apodeme
cs = campaniform sensilla
dcc = distal crenulate carina
csr = caudal setal row
smp = submedial patches
pml = proximomedial lamella
apo = abdominal pulsatile organ
dv = dorsal vessel

cc = caudal chamber
os = ostia
msv = median ventral concavity of T5
Ditr = dorsal intertergal retractor muscle = dorsal internal muscles = 133,144,155
Ite = intertergal extensor muscle = external dorsal muscles = 135, 146, 157
Litr = lateral intertergal retractor muscle = lateral internal dorsal muscles = 134, 145, 156.

Supplementary material 1

Data matrix for phylogenetic analysis
Author: Jonah M. Ulmer
Data type: phylogenetic
Explanation note: Character states fo the examined taxa used in the phylogenetic analysis of the Waterstons evaporatorium.
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Link: https://doi.org/10.3897/jhr.85.67165.suppl1

Supplementary material 2

URI Table of anatomical terms
Author: Jonah M. Ulmer
Data type: morphological
Explanation note: Controlled terminology used within this study referenceable to HAO.
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