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ORIGINAL ARTICLE

A general theory of the complex pronotum morphology of treehoppers in Smiliinae and its relatives (Hemiptera: Membracidae) and its applicability to other subfamilies

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Key words. 3D structure, Centrotinae, Endoiastinae, homology, Membracinae, micro-CT, morphogenesis, pronotum, Stegaspidinae

Abstract. Treehoppers are characterized by exaggerated and three-dimensionally modified pronotal structures, known as “helmets”. The treehopper pronotum has been studied mainly in terms of taxonomic diagnosis for over a century. It has recently been used as a model for studying the origin of evolutionary innovation and morphological diversification. However, the interspecific correspondence of traits, i.e., homology of pronotal parts, remains ambiguous due to (1) the extreme diversity in pronotal morphology and (2) little comparative study focused on homology. These problems hinder tracing the evolutionary history of treehopper pronotal morphology. Here, we investigate the homological relationships of pronotal structures among treehoppers, especially in the subfamily Smiliinae. We first investigate smiliine species, whose pronotal morphology is relatively simple, then expand to species with a more complex pronotum. Our comparative observations using six smiliine species reveal that four traits share the same positions and structures among these species: (1) the humeral angle, (2) the median carina, (3) the starting point of the median carina, and (4) the posterior apex of the pronotum, indicating that these traits are homologous across these species. Based on this homology hypothesis, we generalize the pronotal morphology and propose landmarks to help interpret the diversified pronotum of a clade including Smiliinae and its relatives. Finally, we confirm that this generalization can be applied to other treehopper subfamilies. Our homology-based approach can provide a strategy to recognize the trajectory of pronotal morphology, which is often difficult to trace.

INTRODUCTION

Treehoppers (Auchenorrhyncha: Membracidae) are insects belonging to the order Hemiptera, with approximately 3500 described species typically classified into nine subfamilies (Dmitriev et al., 2024). One of their most notable characteristics is the complex, three-dimensional, enlarged thorn- or horn-like structures on the anterodorsal part of the thorax and extending posteriorly over the body and wings in adults (Buckton & Poulton, 1903; Stegmann, 1998) (Fig. 1). These exaggerated structures are a modified dorsal plate on the prothorax, i.e., “pronotum.” The adult pronotum of treehoppers constitutes a thin, bilayered cuticular structure where the inner and outer cuticle layers are closely attached (Stegmann, 1998; Adachi et al., 2020; Fisher et al., 2020; Sugiura et al., 2024) (Fig. 2). The exaggerated pronotum is formed through a dynamic morphogenetic process that occurs within a simple pouch-like structure surrounded by a single-layered epithelium of the final instar nymph (Adachi et al., 2020; Miranda et al., 2024; Sugiura et al., 2024). Histological observations (Adachi et

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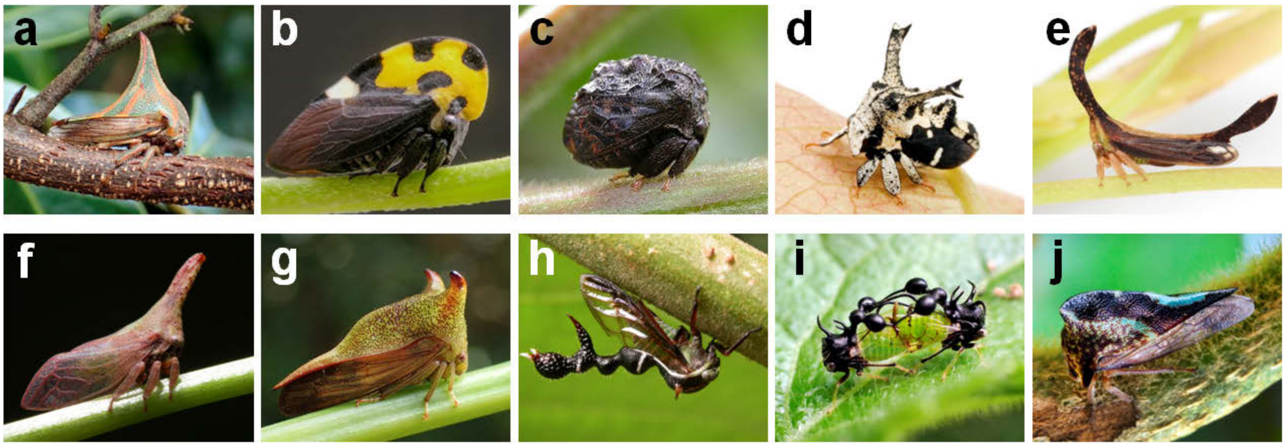

Fig. 1. Various 3D morphologies of treehopper pronota. All species shown here occur in Costa Rica. (a) *Umbonia crassicornis*, (b) *Membacis mexicana*, (c) *Bolbonota* sp., (d) *Notocera* sp., (e) *Cladonota* sp., (f) *Lycoderides serraticornis*, (g) *Hyphinoe vulpecula*, (h) *Heteronotus trinodosus*, (i) *Cyphonia clavata,* (j) *Smilia centralis*.

al., 2020; Sugiura et al., 2024) and gene expression analyses during pronotal formation (Fisher et al., 2020) have revealed similarities in the developmental process between treehoppers' pronotum and wing, making the treehoppers' pronotum an intriguing trait from an evolutionary developmental perspective (Adachi et al., 2020; Fisher et al., 2020), as well as a research model for the origin and evolution of novel morphological features.

The most significant distinction between the pronotum of treehoppers and the insect wing lies in their structural differences. While the wings of most insects exhibit a simple two-dimensional structure, the pronotum of treehoppers possesses a complex three-dimensional architecture (Adachi et al., 2020; Fisher et al., 2020). The modified pronotum is a bilayered structure that extends posteriorly, allowing for pronounced diversification of pronotal morphology, including bending, branching, and other morphological variations unique to each species (Fig. 1). This morphological diversification has been described for over a century, since the early 1900s (Buckton & Poulton, 1903). However, the evolutionary history and transformative process of the pronotal parts still need clarification since multiple terms have been used for putatively the same structures (reviewed by Stegmann, 1998). For example, Godoy et al. (2006) refer to the lateral projection of the pronotum of *Antianthe* as a suprahumeral horn. In contrast, Adachi et

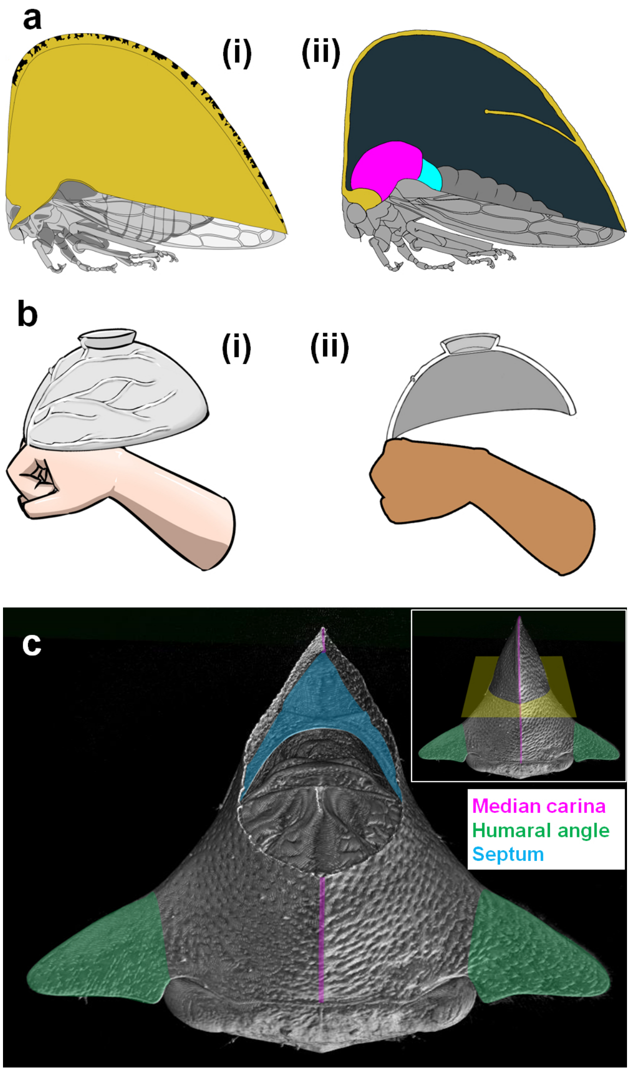

Fig. 2. Morphology of treehopper pronotum. (a-i) Illustration of *Antianthe expansa*. The pronotum (yellow) is prominently developed on the dorsal side. (a-ii) Diagram of *Antianthe expansa*, sagittal plane. The prothorax (T1: yellow), mesothorax (T2: magenta), and metathorax (T3: cyan). The pronotum is an elongated extension of the prothoracic dorsal plate and is only connected to the prothorax (yellow). Note that its inner side is outside of the body. The pronotum is composed of bilayered plate-like structures with a single cuticle layer fused together, and the space between the cuticle layers corresponds to the internal body cavity. Multiple tube-like structures, similar to insect wing veins, are present between the cuticle layers to deliver hemolymph during the molting of nymph to adult (4). (b-i) Conceptual illustration of the pronotum. Imagine a bowl on a fist. If we consider the fist as thorax and arm as abdomen, the bowl represents the pronotum. The bowl (pronotum) has multiple tubes running through it and is connected to the fist at the knuckle (prothorax). (b-ii) Conceptual illustration of the pronotum, sagittal section. The bowl is connected to only a portion of the fist. Its inner side (gray) is outside the body. (c) Micro-CT image of *Antianthe expansa*. The yellow plane of the image on the upper right indicates the sliced part. The membranous structure of the septum is observed, and the median carina extends from near the head posteriorly on the prothorax.

al. (2020) regard a similar projection as a humeral angle. Additionally, the corresponding pronotal structures among species cannot be adequately assessed due to a lack of robust landmarks. The absence of consensus about pronotal homology has impeded our understanding of the evolutionary trajectory of pronotal morphology and has confused the taxonomy and systematics (Stegmann, 1998; Wood, 1993). Since the homology-based approach is essential for interpreting morphological similarities and the evolution of structures in different species (Wood, 1993), more work needs to be done on the homology of pronotal parts through interspecific comparisons across a broad range of treehopper taxa.

In this study, we aimed to identify the morphological correspondence, i.e., homology, of treehoppers' pronotal morphology and to investigate robust landmark traits for tracing pronotal structures among species in terms of relative positions on the pronotum. This procedure should improve the prospects of better understanding adult pronotal morphology among treehopper species since it is difficult to observe the developmental process of the pronotum due to limitations in rearing them. To this end, we carefully observed and described characteristics of the adult pronotum in 14 treehopper species belonging to five subfamilies, including Smiliinae, and compared these with previous descriptions of morphology or development in *Sitctocephala bisonia* (Stegmann, 1998) and *Antianthe expansa* (Adachi et al., 2020), and assessed interspecific consistencies in these traits.

We first focused on three smiliine species with a simple pronotum and found that four traits showed a robust correspondence among them. We also found that these four traits are helpful landmarks in the other smiliine species with more complex pronotal morphologies. Then, we assessed the applicability of these general landmarks to other subfamilies, such as Membracinae, Centrotinae, Stegaspidinae, and Endoiastinae, by observing the characteristics of species within these subfamilies. Finally, we discuss the versatility of the landmarks and propose a hypothetical ground plan of the treehoppers' pronotum that can be used to explain the transformative process of pronotal diversification based on our homology-centered approach.

MATERIAL AND METHODS

Insect collecting

We used six smiliine species, *Antianthe expansa* (Germar, 1835), *Ennya pacifica* (Fairmaire, 1846), *Adippe zebrina* (Fairmaire, 1846), *Vestistilus* sp., *Poppea capricornis* Fowler, 1895, and *Cyphonia clavata* (Fabricius, 1787), four membracine species, *Bolbonota* sp., *Umbonia crassicornis* (Amyot & Serville, 1843), *Alchisme grossa* (Fairmaire, 1846), and *Cladonota* sp., and three species belonging to other subfamilies, *Gargara katoi* Metcalf & Wade, 1965, *Bocydium mae* Flórez-V, 2017, and *Endoiastus caviceps* Fowler, 1896. The treehopper species, except for *G. katoi*, were collected at the University of Costa Rica campus in San Pedro de Montes de Oca (9°56´14˝N, 84°03´03˝W, 1200 m), La Selva Biological Station in Puerto Viejo de Sarapiquí (10°25´50˝N, 84°00´25˝W, 57 m), Quebrada Gonzales in Parque Nacional Braulio Carrillo (504 m), near La Reserva Biológica Alberto Manuel Brenes (10°13´44.48˝N, 84°34´16.03˝W, 764 m), and Estación Biológica Monteverde (10°19´8.30˝N, 84°48´28.95˝W, 1530 m), Costa Rica in November of 2016, 2017 and 2019. All collecting was conducted with the permission of MINAE-SINAC, Costa Rica, resolution numbers SINAC-SE-GASP-PI-R-122-2016, 056-2017-ACC-PI, M-P-SINAC-PNI-ACAT-047-2017 and SINAC-ACC-PI-R-094-2019. The host plants are unknown except for *G. katoi*, adults of which were sampled from a wisteria tree, *Wisteria floribunda*, in Shizuoka-city (35°03´44˝N 138°22´04˝E, Shizuoka, Japan) in 2021. Sampled insects were immediately fixed in 10% formalin and 95% ethanol. We identified and classified genera and subfamilies of sampled species according to Godoy et al. (2006) and Flórez-V et al. (2017). We were unable to identify some individuals to the species level since these individuals might be currently undescribed species.

Criterion of homology

Some criteria to assess the homology of structures have been postulated, such as the shared position of a particular structure or its subparts relative to superior structures, common developmental processes, and/or intermediacy of development (Owen, 1843; Remane, 1952; Wagner, 1989; Wake, 2003). Here, we identify the relationship among the adult pronotal parts in various species according to the biological homology concept based on one of the criteria, i.e., the relative position. This procedure is necessary because it is difficult to compare the developmental process interspecifically due to the inability to rear many treehoppers from egg and early nymphal stages.

Computed tomography (CT)

To observe the inner structure of the adult pronotum, we performed the Micro-CT-scanning, using SkyScan 1172 (SkyScan NV, Belgium). Details of sample preparation methods, scanning, and 3D image construction methods have been described in previous studies (Adachi et al., 2020; Sugiura et al., 2024). In short, fixed samples were dehydrated with an ethanol series and soaked with *t*-butanol before freeze-drying with a FZ-2.5 freeze dryer (Asahi Life Science, Japan). Scanned primary shadow images were reconstructed via NRecon Reconstruction Software Version 1.7.1.0 (Bruker, USA). 3D volume-rendered images were constructed and recorded via the CT Vox Micro-CT Volume Rendering Software Version 3.3.0 (Bruker).

Scanning electron microscope (SEM)

We performed SEM analysis to reveal the surface ultrastructure of the septum of *U. crassicornis*. The fixed samples were freeze-dried using the same method as for Micro-CT. The dried samples were coated with gold (Au) with DII-29010SCTR Smart Coater (JEOL Ltd., Japan). We observed the samples with the SEM, JCM-6000plus (JEOL Ltd.).

Paraffin sections

Paraffin sections were prepared according to the previous studies (Adachi et al., 2020; Sugiura et al., 2024) to observe the fine structures of adult pronota. In brief, the fixed specimens were dehydrated with an ethanol series and cleared with the xylene and paraffin mixture. The paraffin blocks were cut into 6 µm thick sections using a microtome, PR-50 (Yamato Kohki Industrial Co. Ltd, Japan). Hematoxylin-eosin staining was performed to visualize the sections. The stained sections were captured with the digital camera, EOS Kiss X7i (Canon, Japan), attached to the optical microscope CX43 (Olympus, Japan).

Dissection of pronota

To observe the dorsal side of adult pronota, we removed the meso-, metathorax, and abdomen under the binocular/stereo

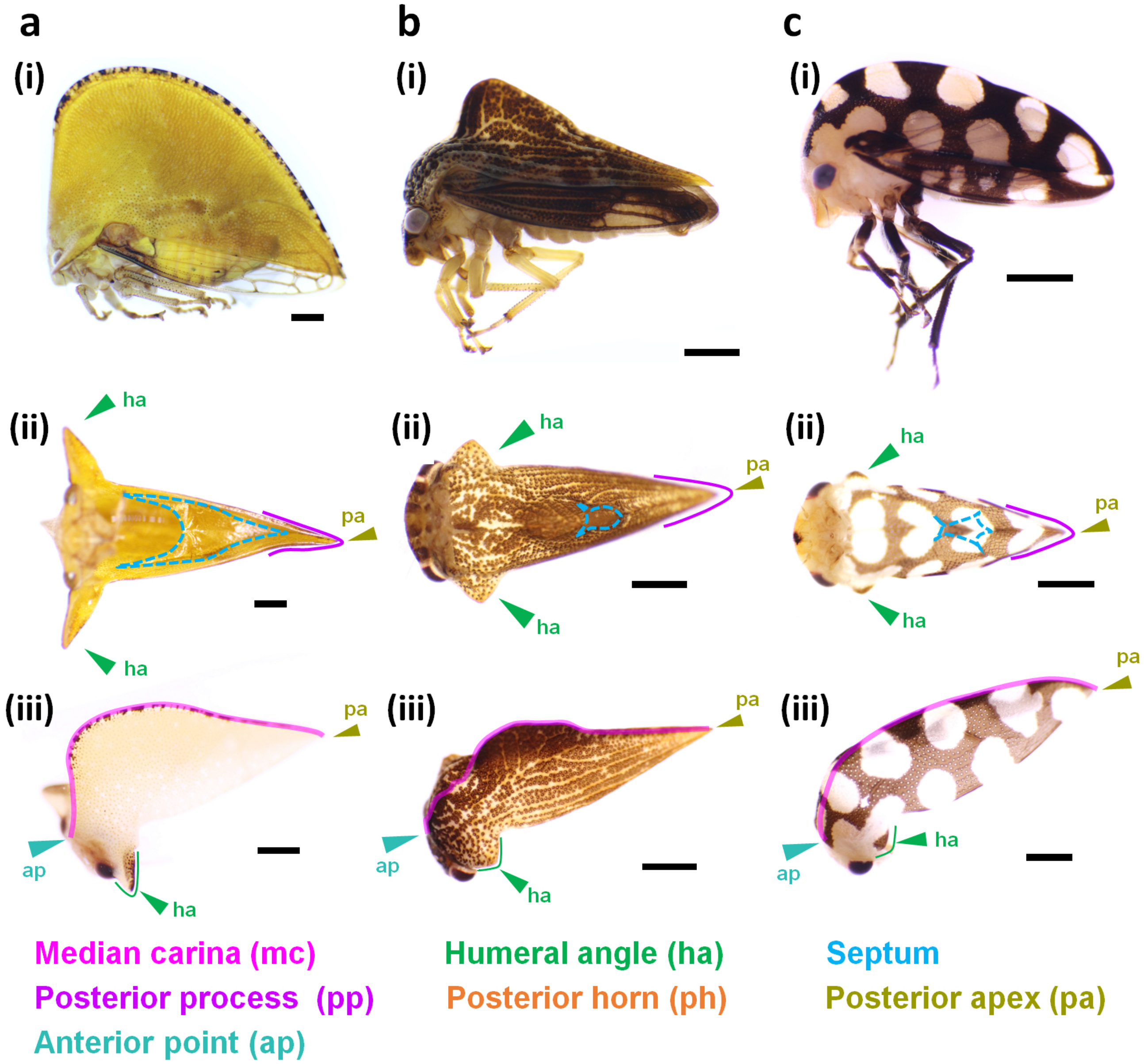

Fig. 3. Gross morphology and structures of pronotum of Smiliini and Polyglyptini. (a) *Antianthe expansa*. (b) *Ennya pacifica*. (c) *Adippe zebrina*. In each species, i, ii, and iii show lateral view, ventral view, and oblique frontal view, respectively. Scale bars: 1 mm.

microscope, SZ61 (Olympus, Japan). Dorsal views of removed pronota were captured with the digital camera, EOS Kiss X9 (Canon), attached to the binocular microscope as described in a previous study (Sugiura et al., 2024).

RESULTS AND DISCUSSION

Generalization of homology of pronotal morphology in Smiliinae and Membracinae

Structures of similar, simple pronotal morphology in three Smiliinae species

The morphology of the treehopper pronotum is drastically diversified between species, even between closely related species (Deitrich et al., 2017), thus complicating a morphological understanding of the pronotum. This morphological diversification impedes a better comparison of pronotal parts among species. To interpret corresponding characters of the pronotal morphology among treehopper species, we first observed and compared the pronota of three smiliine species, *Antianthe expansa* (Tribe: Smilliini) (Fig. 3a), *Ennya pacifica* (Tribe: Polyglyptini) (Fig. 3b), and *Adippe zebrina* (Tribe: Polyglyptini) (Fig. 3c), which possess relatively simple-shaped pronota.

The previous study described the pronotal morphology of *A. expansa* in detail (Adachi et al., 2020). Consistent with the previous description, the pronotum had an evenly convex shape covering the dorsal portion of the thorax and abdomen (Fig. 3a-i). The metopidium of the pronotum sloped precipitously in contrast to a gradual pitch on the posterodorsal margin. Similarly, *A. zebrina* had an evenly convex pronotum (Fig. 3c-i) but flatter than that of *A. expansa*. Unlike these two species, the pronotum of *E. pacifica* exhibited an undulating shape, which had a dorsally protruded central region (Fig. 3b-i). Therefore, the gross pronotal morphology differed among these species.

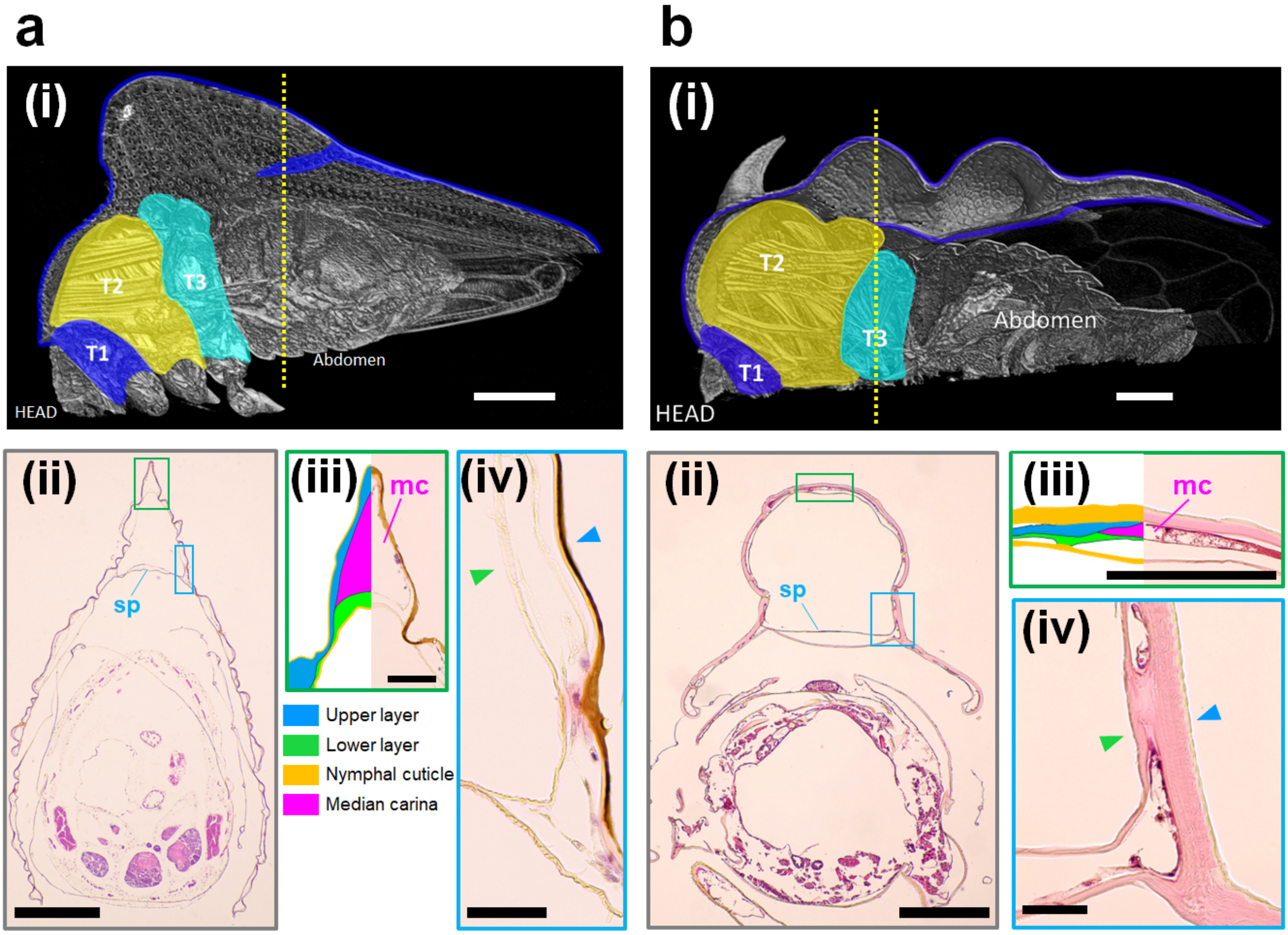

Fig. 4. Pronotum structures of Smiliinae with modified (more complex) pronotum shape. (a-i) Micro-CT image of sagittal plane of *Ennya pacifica*. The contour of the pronotum is different from *Antianthe expansa*, but the position of the septum is nearly the same. (a-ii) Paraffin section of the dashed line in (a-i). The overall shape is similar to that of *A. expansa*, but the surface texture is more rough and uneven. (a-iii) Magnified image of the green square area in (a-ii), mc: median carina. (a-iv) Magnified image of the cyan square area in (a-ii). Cyan arrowhead points to upper layer, and green arrowhead points to lower layer. (b-i) Micro-CT image of the sagittal plane of *Poppea capricornis*. (b-ii) Paraffin section of the dashed line in (b-i). The septum, which was difficult to discern in the sagittal sections, more clearly observed. (b-iii) Magnified image of the green square area in (b-ii), mc: median carina. (b-iv) Magnified image of the cyan square area in (b-ii). Scales: 1 mm (a-i, b-i), 0.5 mm (a-ii, b-ii), 0.05 mm (a-iii, a-iv, b-iii, b-iv).

Other traits of the pronotum were similar in position and shape among the three species, in contrast to the differences in gross morphology. In all three species, the pronota possessed a pair of ventromost projections in the anterolateral part of the pronotum wall between the eye and wing (Fig. 3a-ii, 3b-ii, and 3c-ii). The shape of the anterolateral protrusion was different in size between the three species. In *A. expansa,* the shape was vastly extended distally and directed slightly forward, similar to an ox horn. *Adippe zebrina* possessed a slight ridge-like protrusion (Fig. 3c-ii), while the corresponding structure of *E. pacifica* was intermediate between the other two (Fig. 3b-ii). Although the size and shape differed interspecifically, the anterolateral protrusions shared the same relative position on the pronotum. The interspecific consistency in the position of anterolateral protrusions of the pronotum in these species strongly supports a homologous relationship. Moreover, the protrusions of these species consisted of an epithelial layer of the pronotum wall rather than a mere cell mass, which also supports the homology of these structures (Fig. S1). Due to differences in shape and size between groups these protrusions have been referred to with different terms, such as humeral angle (Stegman, 1998), suprahumeral horn (Godoy et al., 2006), and humeral horn (Adachi et al., 2020). However, in our homology-based perspective, the paired protrusions located on the anterolateral region of the pronotum between the eye and the wing are reasonably described by the term humeral angle. For example, the large protrusions found in *A. expansa* are more adequately referred to as "long humeral angles" (Evangelista et al., 2017a) or "pronounced humeral angles" (Plummer, 1938) rather than "suprahumeral horns" (Godoy et al., 2006).

In all three species, a tubular lumen ran through the dorsal margin of the pronotum beginning at the contact zone with the prothoracic wall (Figs 3a-iii, 3b-iii, 3c-iii, 4a, and 4b). This lumen is called a median carina (Adachi et al., 2020). The median carina contained a single epithelial layer, like insect wing veins in general, but unlike a dorsal-ventrally closed epithelium or bilayered structure of the pronotal walls (Fig. 4). The similarity of epithelial structure and relative position on the pronotum indicate that the median carina is homologous among the three species. The median

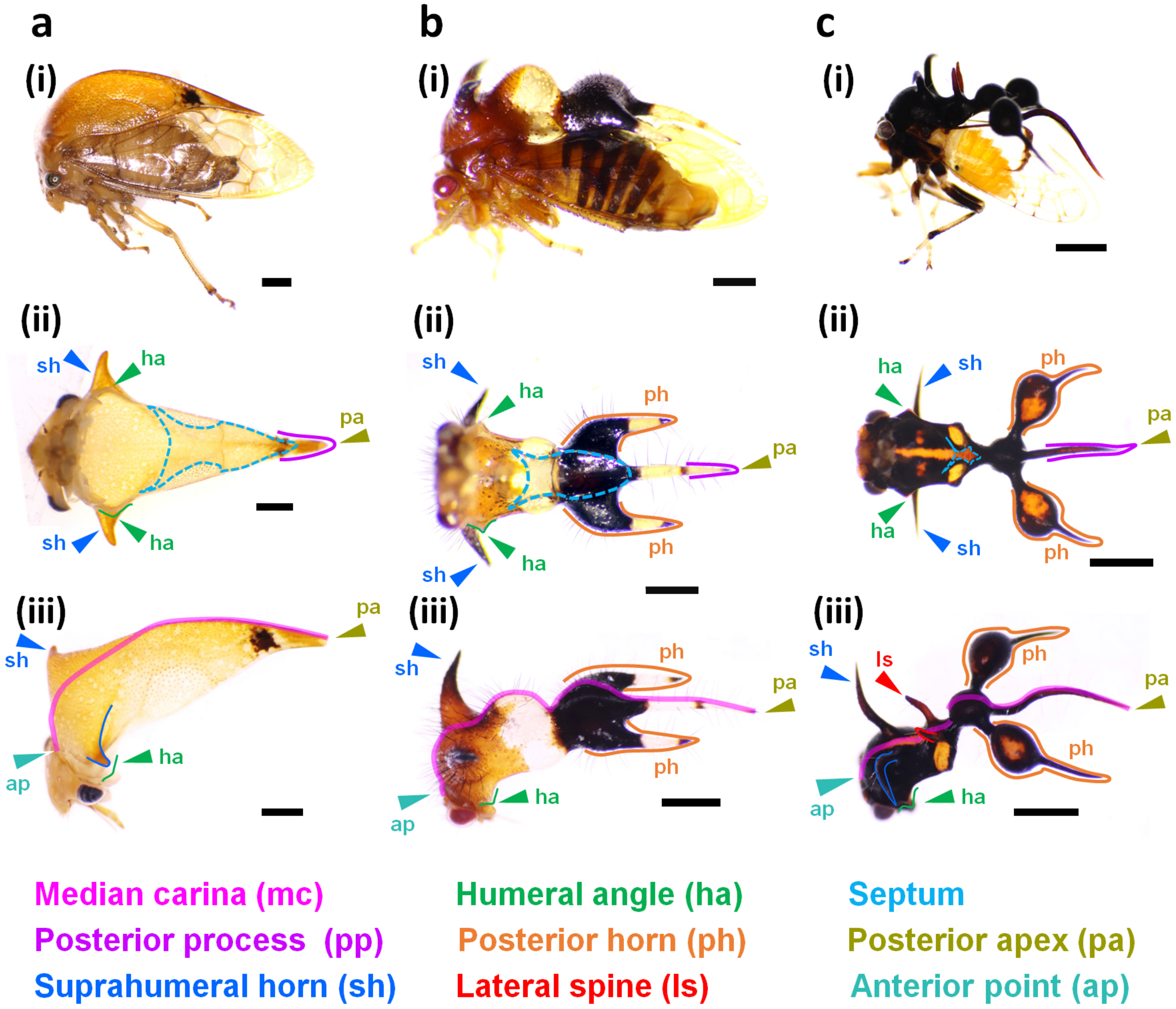

Fig. 5. Gross morphology and structures of pronotum of Ceresini. (a) *Vestistilus* sp. (b) *Poppea capricornis*. (c) *Cyphonia clavata*. In each species, i, ii, and iii show lateral view, ventral view, and oblique frontal view, respectively. Scale bars: 1 mm.

carina terminated at the posterior apex in all three species (Fig. 3a-iii, 3b-iii, and 3c-iii). Thus, we adopted the posterior apex as a homologous character among species. Additionally, the left and right sides of the inner pronotal wall were bridged by a plate-like structure (Figs 3a-ii, b-ii, c-ii, 4a, and 4b). This bridge has been called a septum (Stegmann, 1998). The shape and size of this bridge differed among species: a broad triangular plate in *A. expansa* (Fig. 3a-ii), a small oval plate in *E. pacifica* (Fig. 3a-ii), and a small pentagonal plate in *A. zebrina* (Fig. 3a-ii). Although the septum was interspecifically different in size and shape, the relative position and structural features were robustly shared among the three species: an outgrowth from the posterolateral wall, dorsal to the posterior apex, and consisting of a bilayered cuticle. The septum thus showed correspondence among these species. The vein-like epithelial lumen was also found in the lateral wall of the pronotum in all three species, which has been referred to as the lateral carina (Adachi et al., 2020). However, the number and extent of the lateral carinae varied among species: *A. expansa* had two pairs of lateral carinae, one running along the ventral edge and the other along the lateral wall to the septum (Adachi et al., 2020); *E. pacifica* exhibited an even greater number, one running along the ventral edge and others being complexly branched (Fig. 4b). Thus, this positional and structural diversification makes it challenging to trace the corresponding character.

The comparative and homology-based observation of some pronotal parts corresponded morphologically: (1) the humeral angle, (2) the median carina, (3) the starting point of the median carina, (4) the posterior apex of the pronotum, and (5) the septum. The morphological diversification among the three species can mainly be explained as transformations of these traits, e.g., the longer or shorter humeral angle and the smooth or undulating margin between the initial and terminal part (= posterior apex) of the median carina. Hence, these traits can be useful as putative landmarks to trace the evolution of pronotal morphology if these traits are robustly shared among other smiliine species with more complex pronota.

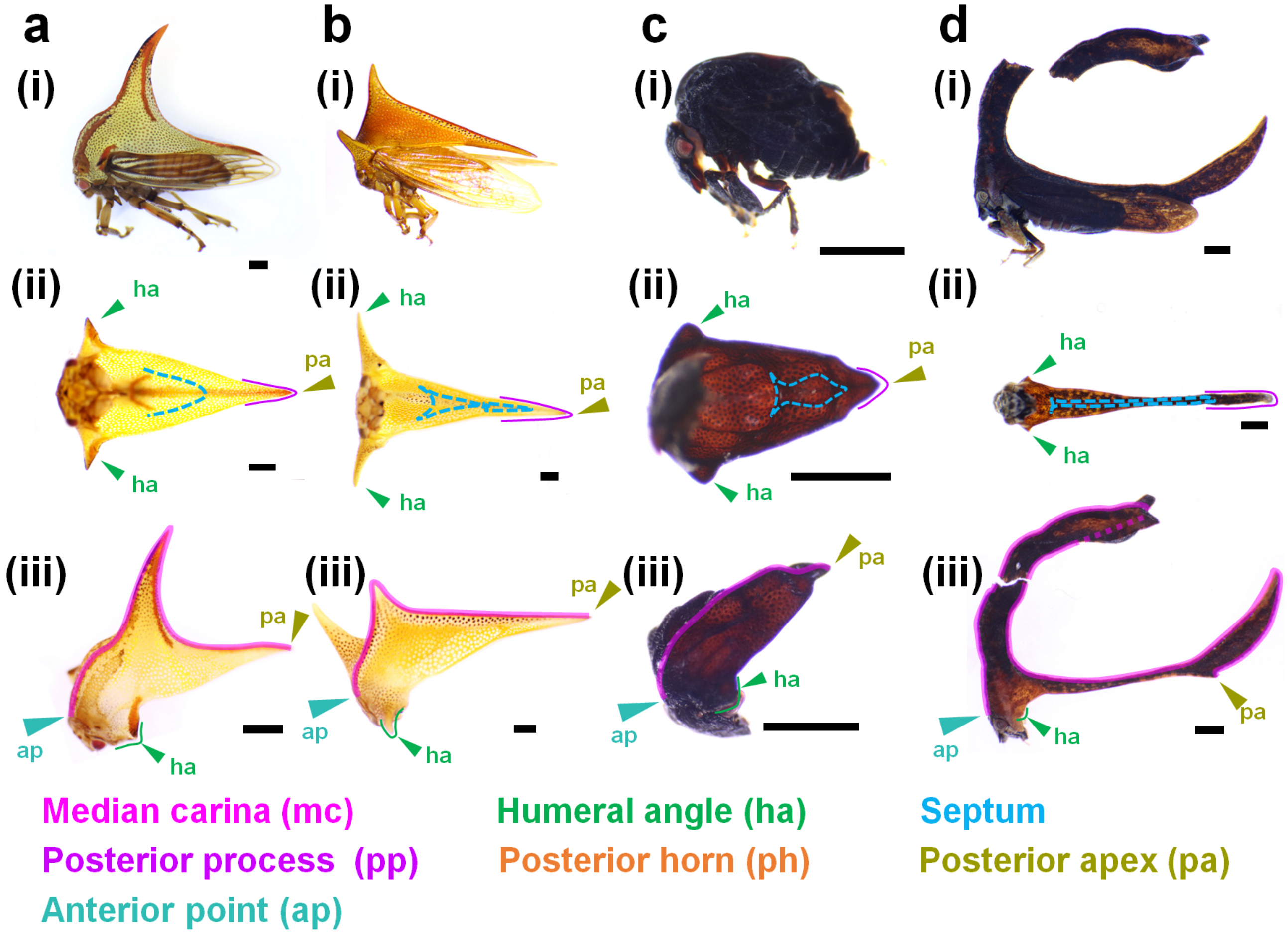

Fig. 6. Gross morphology and structures of pronotum of Membracinae. *Umbonia crassicornis*, (b) *Alchisme grossa*, (c) *Bolbonota* sp., (d) *Cladonota* sp. In each species, i, ii, and iii show lateral view, ventral view, and oblique frontal view, respectively. Scale bars: 1 mm.

Structures of more complex pronotal morphology in other Smiliinae species

The three smiliine species mentioned above belong to closely related tribes: Smiliini (*A. expansa*) and Polyglyptini (*E. pacifica* and *A. zabrina*) (Dietrich et al., 2001, 2017). Also, the pronotal structures of these species are relatively simple. To examine the applicability of these characters to a broader range of taxa and more complex pronota, we then investigated these traits in species with more complex pronota, belonging to a more distantly related tribe, Ceresini: *Vestistilus* sp., *Poppea capricornis*, and *Cyphonia clavata* (Fig. 5). Recent molecular-based phylogenetic analyses have reconstructed the monophyly of Smiliini, Polyglyptini, and Ceresini (Evangelista et al., 2017a, b). Moreover, these studies suggest that Smiliini and Polyglyptini are clustered into a derived group of this monophyletic clade. In contrast, Ceresini is phylogenetically located in a relatively basal group (Evangelista et al., 2017b).

Vestistilus sp. had an evenly convex pronotum with an elongated posterior region, i.e., posterior process, and with an elongate, buffalo horn-like, suprahumeral horn on each side (Fig. 5a). *P. capricornis* possessed an undulating, bimodal pronotum with suprahumeral horns and a posterior process. However, the posterior process was trifurcated, with two laterally extended posterior horns and one centrally elongated projection (Fig. 5b). The pronotum of *C. clavata* is even more complex, having paired projections referred to as lateral spines (Godoy et al., 2006), in addition to the suprahumeral horn and a trifurcated posterior process where the basal region of the posterior horns is swollen (Fig. 5c). Despite this diversification in pronotal gross morphology, a paired protruding structure was found in the anterolateral, lateral margin of the pronotum wall between the eye and the wing in all these species, which corresponds to the humeral angle of the former three species in terms of relative position (Fig. 5). Note that the "lateral margin" in the specialized pronotum of treehoppers may not necessarily be homologous to the same term as used for the pronotum in other Hemiptera. In this paper, the "lateral margin" of the treehopper pronotum refers to the ventral edge of the pronotum, following the terminology used by Godoy et al. (2006). The suprahumeral horn in these species grew from the more dorsal portion and coexisted with the "true" humeral angle, suggesting that the suprahumeral horn does not correspond to the humeral angle in these species. The median carina ran through the dorsal margin of the pronotum from the contact zone with the body wall. Considering the posterior termination of the median carina, the posterior apex corresponded to the edge of the posterior process in *Vestistilus* sp., the end of the central projection

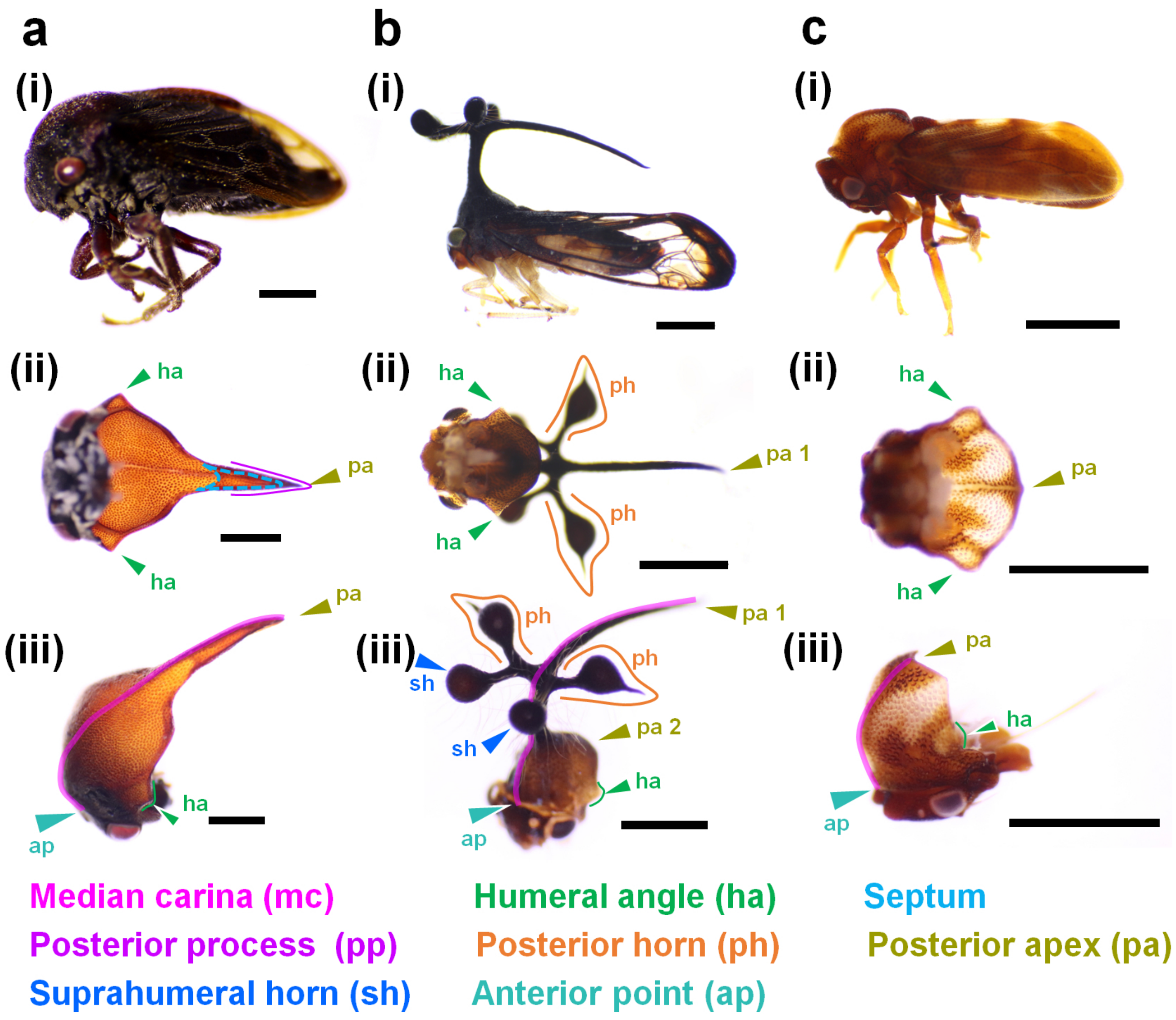

Fig. 7. Gross morphology and structures of pronotum of Centrotinae, Stegaspidinae and Endoiastinae. (a) *Gargara katoi*, (b) *Bocydium mae*, (c) *Endoiastus caviceps*. In each species, i, ii, and iii show lateral view, ventral view, and oblique frontal view, respectively. Scale bars: 1 mm.

of the trifurcation in *P. capricornis* and *C. clavata*. The septum was present in a similar position in all six species despite differences in size and shape.

All six studied smiliines shared relative position on the pronotum and structural characters of the five traits, indicating that these traits are homologous among Smiliini, Polyglyptini, and Ceresini, at least in the homology concept where corresponding characters are produced by the same morphological and developmental constraints, and originate from a common ancestor (Remane, 1952: Cryan et al., 2000). Considering previous descriptions, this homology hypothesis could be applied to other Ceresini species, such as *Stictocephala bisonia* (Stegmann, 1998), since the positions of these five traits are the same as those in the six species. Likewise, descriptions of the pronotum of smiliine species belonging to other tribes, including Amastirini, (e.g., *Amastris comarapa*) (Evangelista & Sakakibara, 2007), Telamonini, e.g., *Heliria cristata* (Wallace, 2011), and Actalini, e.g., *Tectiforma guayasensis* (McKamey, 2023), show similar structures in the identical location as those of our species. However, since the septum is lacking in some smiliines (Fig. S2a) and, where present, it varies drastically in its range and shape among species, it may be unsuitable for evaluating landmarks in pronotal morphology. Hence, we propose that the following four traits can be used to generalize landmarks in the transformative process or evolution of the pronotum in the investigated tribes.

1. *Humeral angles*, a pair of ventromost protrusions growing from the anterolateral area of pronotum, located between the eye and the wing, consisting of a bilayered cuticle.

2. *Median carina*, a vein-like structure running along the dorsal median line from the contact zone between the prothoracic wall and pronotum, consisting of a single layer of epithelium.

3. *Anterior point*, a median point of the anterior margin at the cuticular contact between the prothoracic wall and

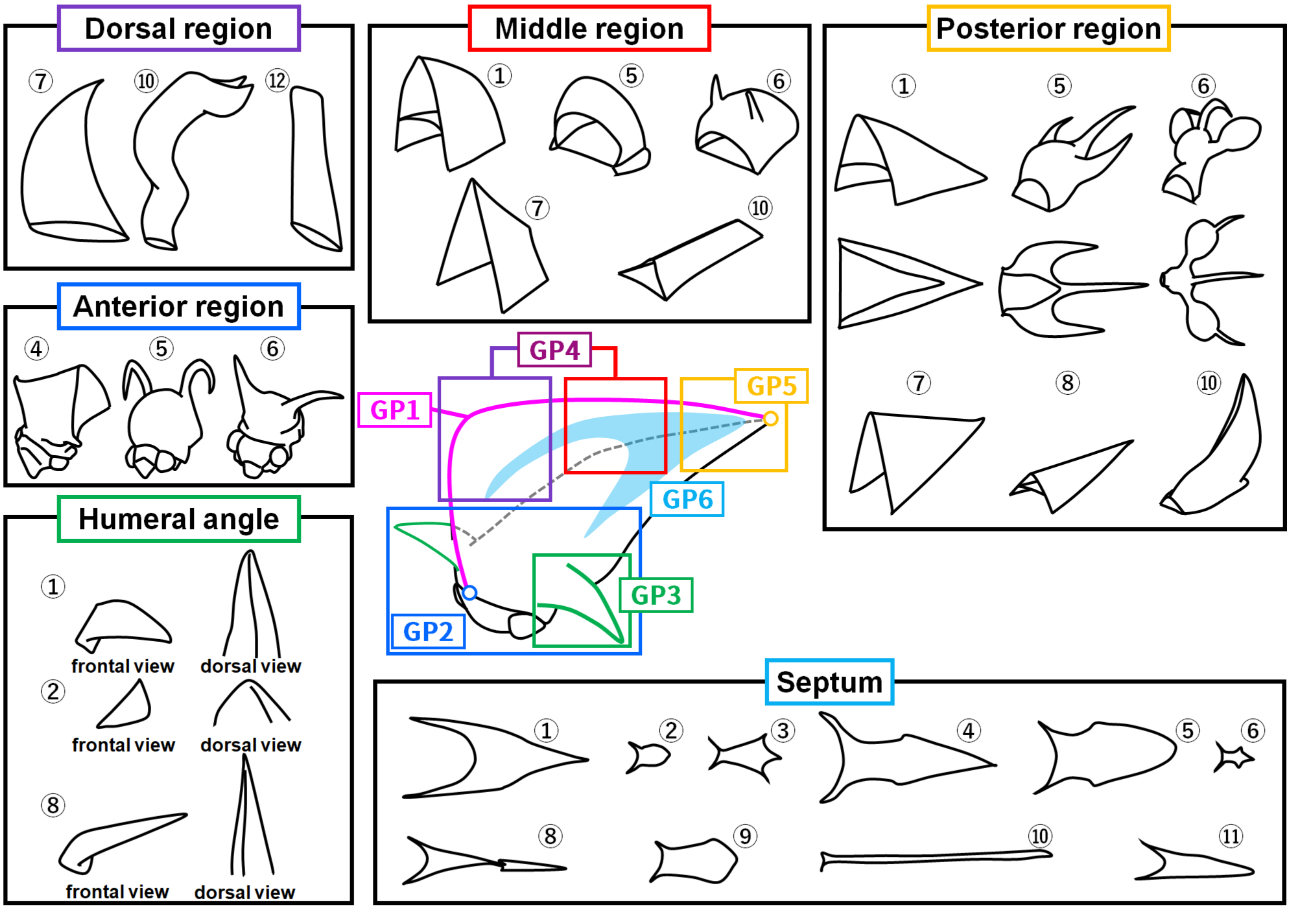

Fig. 8. Schematic diagrams of homologous characters in selected treehopper species. Treehopper pronotum divided into different regions, with the shape of each region illustrated with representative species having distinctive features. Anterior region: The shape from the head to the base of the pronotum. Dorsal region: The shape of the portion extending distally on the dorsal side of the pronotum. Middle region: The shape of the intermediate portion of the pronotum. Posterior region: The shape of the distal portion of the pronotum. Figs 3 and 5–7 are provided for the oblique frontal view and ventral view. Humeral angle: Figures are provided for frontal view and dorsal views. Septum: The shape of the septum as viewed from the ventral side. 1. *Antianthe expansa*, 2. *Ennya pacifica*, 3. *Adippe zebrina,* 4. *Vestistilus* sp., 5. *Poppea capricornis*, 6. *Cyphonia clavate*, 7. *Umbonia crassicornis,* 8. *Alchisme grossa*, 9. *Bolbonota* sp., 10. *Cladonota* sp., 11. *Gargara katoi*, 12. *Bocydium mae*.

pronotum, i.e., the starting point of the pronotum and its median carina.

4. *Posterior apex*, a posterior termination of the median carina and a posterior end point of the posterior process.

Based on this homology hypothesis, some subparts of the pronotum can be traced among these related species. For example, the suprahumeral horn protrudes from the anterolateral wall. It is located dorsal to the humeral angle in all species, suggesting that the suprahumeral horn is homologous among these six species. However, the lateral spine of *C. clavata* does not correspond to the suprahumeral horn of other species in view of the relative position to the humeral angle. Thus, the lateral spine is likely an evolutionary novelty in *C. clavata* or its relatives since this structure is not present in the other species (Godoy et al., 2006). The posterior horns of *P. capricornis* and *C. clavata* are different in shape; however, they consistently grow from the intermediate area of the lateral margin between the posterior apex and humeral angles, suggesting that the posterior horns are homologous structures in the two species. Therefore, the landmarks we formulated can help identify homologous relationships between morphologically diversified structures within a clade including Ceresini, Smiliini, and Polyglyptini. More inclusive comparisons, including morphometrics, will help infer an evolutionary history of complex pronotal structures in the clade.

Structures of the pronotum in the subfamily Membracinae

The smiliine tribes investigated in this study can be clustered into a clade. However, in the last two decades the monophyly of the classical Smiliinae subfamily has no longer been supported by morphology- and molecular-based phylogenetic analyses (Dietrich et al., 2017; Evangelista et al., 2017a, b). Almost all phylogenetic studies showed that tribes of Smiliinae, Darninae, and Membracinae are nested in a clade (Dietrich et al., 2017; Evangelista et al., 2017a, b). To investigate the applicability of the landmarks established for the above smiliine tribes to other related groups, we carefully observed the pronotum of four species belonging to the subfamily Membracinae: *Bolbonota* sp. (Tribe: Membracini), *Umbonia crassicornis* (Tribe: Hoplophorionini), *Alchisme grossa* (Tribe: Hoplophorionini), and *Cladonota* sp. (Tribe: Hypsoprorini) (Fig.

6). In all these species, a pair of protrusions was found at the same position as the humeral angle in smiliine species despite their size differences: long and horn-like in *A. grossa*, a rudimentarily ridge-like structure in *Bolbonota* sp. and *Cladonota* sp., and an intermediate-sized structure in *U. crassicornis* (Fig. 6). In all these species, the epithelial layer also ran through the median line of the pronotum from the contact zone with the body wall on the anterior margin to the posterior end, i.e., the posterior apex (Fig. 6). These consistencies of the landmark traits established for smiliine species ensure that they are also helpful landmarks in Membracinae.

The septum was also detected as a bridge between the left and right sides of the inner pronotum wall in all these membracine species. However, as in the case of smiliines, the septum morphology differed strikingly between species: a small pentagonal plate in *Bolbonota*, a two-tiered structure with two triangles in *A. grossa*, and an elongated plate in *Cladonota* (Fig. 6b-ii, 6c-ii, and 6d-ii). The most different septum was observed in *U. crassicornis* which was rudimentary, with only a limited area exhibiting a folded bilayered structure (Figs 6a-ii and S2b). Therefore, the septum is unsuitable as a landmark trait in Membracinae. Hence, the landmarks for the smiliine species can be applied to the pronotal structures of Membracinae.

Based on the relative positions of the landmarks, the origin of complexities in the membracine pronota can be interpreted as follows. *U. crassicornis*, *A. grossa*, and *Cladonota* sp. exhibited an upward protrusion at the anterior or middle region of the pronotum (Fig. 6a-i, 6b-i, and 6c-i). This protrusion in the three species had the median carina running along the medial contour (Fig. 6a-iii, 6b-iii, and 6d-iii), suggesting that this protrusion can be interpreted as a modification of the dorsomedial contour of the pronotum rather than a novel tubercular process on the dorsal surface of the pronotum. Although the homology of the protrusion among membracine species is uncertain due to the small number of studied species, the interspecific similarity of its growth area suggests a sharing of a morphological field. Additionally, *Cladonota* sp. possessed an upward process at the most posterior portion. Since the median carina also ran through the surface of this process (Fig. 6d-iii) and extended to the distal region, i.e., the posterior apex, the process can be regarded as the outgrowing dorsoposterior portion proximal to the posterior apex. As this process does not exist in most other Membracinae and is present in many other *Cladonota* species (Flynn, 2019), this process may be an apomorphy of the genus *Cladonota*, though this requires further investigation.

Expanding the landmarks to distantly related subfamilies Centrotinae, Stegaspidinae and Endoiastinae

Finally, we examined whether the landmarks potentially apply to more distantly related subfamilies, for example, Centrotinae, Stegaspidinae, and Endoiastinae (Fig. 7). In *Gargara katoi* (Centrotinae), *Bocydium mae* (Stegaspidinae), and *Endoiastus caviceps* (Endoiastinae), the humeral angle, median carina, and anterior point exist at the same position as in Smiliinae and Membracinae (Fig. 7). Therefore, even though we observed only a single species per subfamily, this result suggests that these three traits are potentially suitable landmarks in the treehopper pronotum for these subfamilies.

Unlike the explicit recognition of the posterior apex in *G. katoi* (Fig. 7a), the largely different pronotal gross morphology of *B. mae* was difficult to assess (Fig. 7b). Two possible candidates for the posterior apex of *B. mae* are the posterior tip of the dorsally extending process (posterior apex 1) and the ventral posterior tip of the helmet (posterior apex 2) (Fig. 7b-ii and 7-iii). In the movie of molting *Bocydium* sp. (Adachi et al., 2020, Suppl. movie S2), the posterior end of the nymphal prothorax corresponds to posterior apex 1 of the adult helmet. In *A. expansa* and *P. capricornis*, the posterior apex of the adult helmet was found to be the posterior end of the nymphal prothorax (Adachi et al., 2020; Sugiura et al., 2024), and in *B. mae*, posterior apex 1 is considered to be the "true" posterior apex. In *E. caviceps*, a clear posterior process could not be confirmed due to the rounded posterior region. However, the posterior apex is defined as the posterior terminus of the median carina, so we were able to confirm it in this species (Fig. 7c-ii and 7c-iii). Hence, the posterior apex is likely a useful landmark in the three subfamilies. Another posterior end point of *B. mae* (Fig. 7b-iii, pa2) seems unlikely to be the posterior apex and may be interpreted as a partial fusion of the left and right lateral margins of the pronotum.

The septum was distinguishable in *G. katoi* at a position similar to the other subfamilies (Fig. 7a-ii). However, no distinct septum was observed in *B. mae* and *E. caviceps* (Fig. 7b-ii and 7c-ii), suggesting that the septum is an unsuitable trait for a landmark in these subfamilies.

B. mae had two paired horn-like structures on the spine (Fig. 7b). Since each side of the anterior and posterior processes was fused in the proximal region, the structure was seen as a paired bifurcating horn. This horn grew from the anterior part of the line connecting the anterior point and posterior apex and above the humeral angle (Fig. 7b-iii). This position bore a striking resemblance to the growing point of the suprahumeral horn in some smiliine species (Fig. 5). Although the suprahumeral horn in the smiliine species and the horn-like structure in *B. mae* could be a homoplasy rather than a homology, based on the distant relationship between these groups, it is plausible that these two horn-like structures share the same morphogenic field. Similar horn-like structures have been described in the genus *Elaphiceps* (Centrotinae) (Buckton & Poulton, 1903). Likewise, the dorsal protrusion of Membracinae may share the same morphogenic field (see previous section). It is speculated that limited morphogenic fields might be evolutionary hotspots where structures such as horns and protrusions repeatedly emerged, underpinning a part of the morphological diversity of the treehopper pronotum. Developmental and phylogenetic comparisons are needed to test this hotspot concept.

CONCLUSION AND FUTURE PERSPECTIVE

In this study, we examined the homology of the treehopper pronotum in terms of the relative positions of each character. We proposed homologous landmarks for treehopper pronotal morphology, primarily focusing on some smiliine and membracine species, and suggested that these landmarks are likely to apply to more distantly related subfamilies (Centrotinae, Stegaspidinae, and Endoiastinae). Our homology-based landmarks should help us understand the evolution of the complex pronotal morphology which has previously been untraceable.

Recent phylogenetic studies support the monophyly of the group consisting of Smiliinae, Membracinae, and Darninae (hereafter, SMD clade) (Evangelista et al., 2017b). In this study, we established robust landmarks for pronotal comparison among smiliine tribes, Smiliini, Polyglyptini, and Ceresini, and membracine tribes, Membracini, Hoplophorionini, and Hypsoprorini. Furthermore, recent descriptions of the pronotal structures of Darninae species, such as *Nasconia nanica*, drew similar traits in identical positions to those of our species (González-Mozo & Ware, 2023). Likewise, the relatively early-diverging groups of the SMD clade, such as Acutalini and Micrutalini, possess similar traits (McKamey, 2023; Flynn & Wheeler Jr., 2016). Although more information on these groups would be needed to determine the structural correspondence between Darninae and the other two related subfamilies, the current descriptions support our landmark traits as existing in a common ancestor of the SMD clade. Also, the septum can be observed in the smiliine and membracine species investigated here. Procyrtini, a putative early-diverging tribe of the SMD clade (Godoy et al., 2006; Dietrich et al., 2017), possesses a smooth, small, evenly convex pronotum. Integrating these inferences, we propose the following traits as groundplans (GP, Fig. 8) for explaining transformative processes of the pronotal morphology in the SMD clade.

GP1: The smooth, small, evenly convex gross morphology with the median carina running through the dorsal medial line.

GP2: The anterior point, the contact zone between the pronotum and prothoracic wall, i.e., the starting point of the median carina.

GP3: The humeral angle growing from the anterodorsal area of the ventral region between the eye and the wing.

GP4: The smooth middle area without any spine.

GP5: The unbranched posterior portion with the posterior apex, i.e., the posterior endpoint of the pronotum and the terminal of the median carina.

GP6: The septum, i.e., the bilayered bridge between the left and right inner walls.

The diversification of the pronotum in the SMD clade can be interpreted as some modification of the above ground plan (Fig. 8), e.g., a change in the size of the landmark traits, an enhancement of the height of the convexity, a protrusion of the anterior part of the line connecting the anterior point and posterior apex, and a bulging out of the intermediate area of the lateral margin between the posterior apex and humeral angle. Considering the consistency of the landmarks not only in the SMD clade but also in other subfamilies examined, it is possible that the ground plan outlined here might apply to the entire Membracidae family. To test the versatility of the landmarks and ground plan and to verify the homologies of the landmarks between subfamilies, a comparison of the developmental processes of adult pronotum formation across species and phylogenetic mapping using more inclusive information from a wide array of taxa will be needed in order to shed light on the evolutionary history of the complex morphology of the treehopper pronotum.

DECLARATIONS

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests. The authors declare that they have no competing interests.

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Authors' contributions. Study design: KS, YC and HG. Sample preparation and observation for micro-CT, SEM and paraffin section: KS, TT, HA, JH, KM and HG. Contribution to field sampling: HA, HG, SK, KN and PH. Live treehopper photos: KN. Figure preparation: KS, TT and HG. Manuscript preparation: KS, YC and HG. All authors reviewed, made corrections, and approved the manuscript.

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Supplementary Figs S1 and S2 follow on next pages.

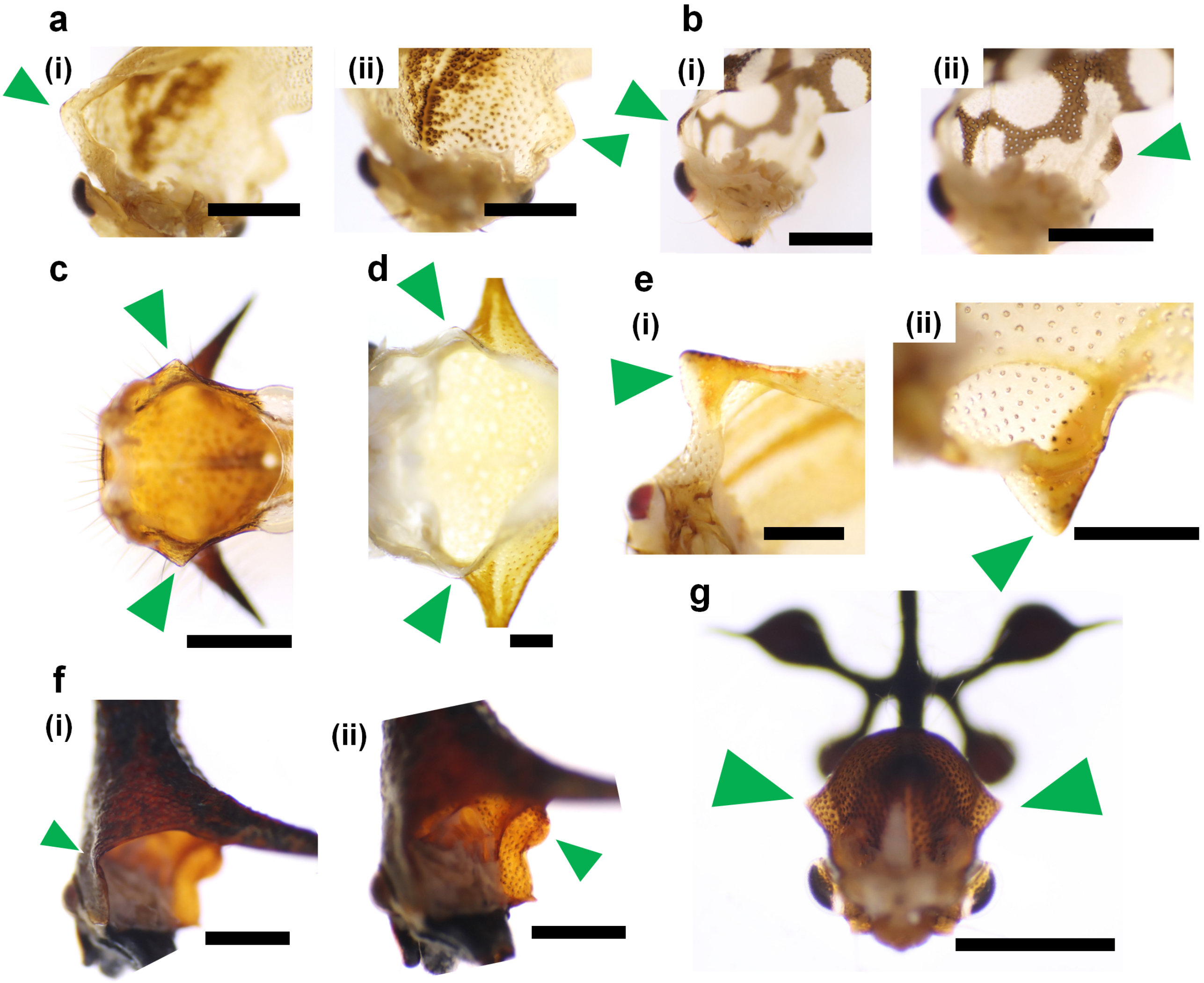

Fig. S1. Humeral angle in the ventral view. (a) *Ennya pacifica*. (b) *Adippe zebrina*. (c) *Poppea caprinornis*. (d) *Venstistils* sp. (e) *Umbonia crassicornis*. (f) *Cladonota* sp. (g) *Bocydium mae*. Inside of left (i) and right (ii) humeral angle. Scale bars: 1 mm. Arrowheads indicate humeral angles.

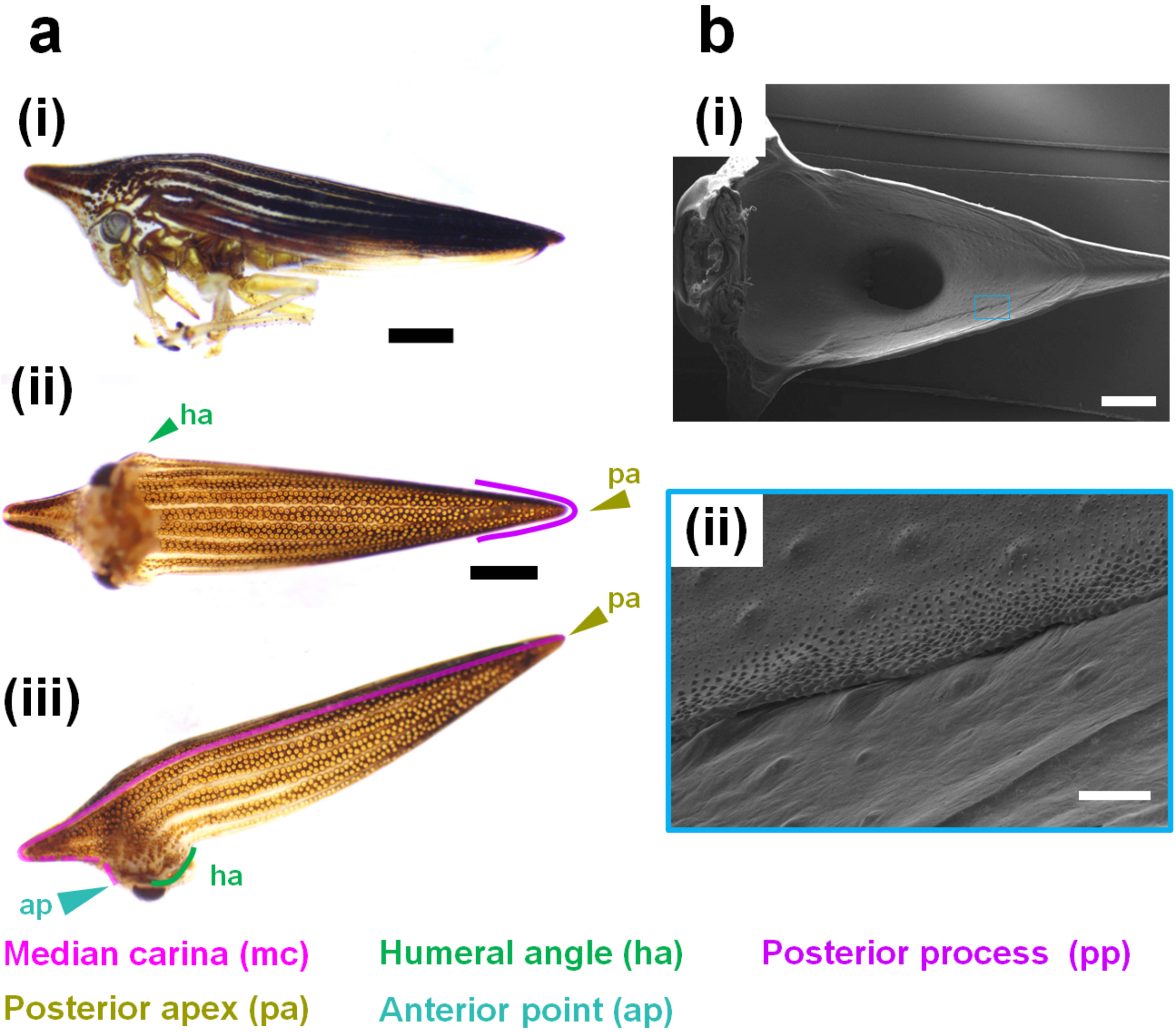

Fig. S2. Structure of pronotum of species without or with the septum. (a) *Polyglypta* sp. (a-i) Elongate shape with a horn-like structure in the front. (a-ii) This species lacks an obvious septum. (a-iii) Humeral angle and median carina. Scale bars: 1 mm. (b) *Umbonia crassicornis* (b-i) SEM image of pronotum in the ventral view. Scale bar: 1 mm. (b-ii) Enlargement view of the blue square area in (b-i). Scale bars: 0.1 mm.