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Phylogeny of Tortricidae (Lepidoptera) a Morphological Approach with Enhanced Whole Mount Staining Techniques

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Phylogeny of Tortricidae (Lepidoptera): A morphological approach with enhanced whole mount staining techniques

By

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A comprehensive review of whole mount staining revealed an enhanced staining method to improve visibility of morphological structures. Ultimately, five factors vital to stain quality were identified, with pH being a primary factor. Phylogenetic relationships of all 22 tribes of Tortricidae, representing 57 genera and 78 species (distributed in Neotropical, Nearctic, Palearctic, Oriental, and Australian regions), were investigated based on 52 morphological characters, including incorporation of 27 novel non-traditional characters of the endo- and exoskeleton. The phylogenetic analysis yielded five equally parsimonious trees (length 389 steps, CI=0.2571, RI=0.7051), a strict consensus (length 392 steps, CI=0.2551, RI= 0.7021) of which produced two trichotomies. These results reinforced those from previous molecular analyses (Fagua et al., 2016; Regier et al., 2016) with some disagreement, consistent with historical conclusions made based on morphology. Mapping character distribution onto a recent molecular based phylogeny (Regier et al., 2012) revealed promising characters for subfamily and tribal delimitation.

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CHAPTER I

LITERATURE REVIEW

Introduction

The Tortricidae are among the largest families of the microlepidoptera, representing 1,077 genera and about 10,000 species worldwide (Baixeras et al., 2010). The tortricids are comprised of numerous economically important pests (e.g. spruce budworm, pine tip moth, and the oriental fruit moth) (Chapman & Lienke, 1971; van der Geest & Evenhuis, 1991; Zhang, 1994), as well as biological control agents (e.g. sulphur knapweed moth, and the ragweed borer moth) (Dhileepan, 2003; Müller et al., 1988; Powell et al., 2000). In addition to being the third most economically important group of Lepidoptera, the Tortricidae include many well-known model organisms that have been used for the study of pheromones, wing pattern evolution, and genetics (Baixeras, 2002; Brown & Powell, 1991; Roe et al., 2010; Roelofs & Brown, 1982). Although distributed world wide, the bulk of tortricid diversity exists in the New World tropics (Regier et al., 2012).

The family includes three subfamilies, the Olethreutinae, with 4,417 species, the Tortricinae, with 4,176 species, and the Chlidanotinae, with 288 species (Regier et al., 2012). Monophyly of the family is supported with molecules (Regier et al., 2012), but also with morphology (Horak & Brown, 1991). Regier et al. (2012) examined 19 of the 22 tribes and found support for the monophyly of the Tortricinae and the Olethreutinae,

however their sampling was insufficient for six tribes, and there was no resolution or low support for several other tribes. Morphological analyses have not yielded compelling evidence of the phylogenetic relationships within the subfamilies, and the 22 tribes contained within them. Horak and Brown (1991) used traditional morphological characters to develop their estimate of phylogenetic relationships. Traditional characters include wing pattern and venation, characters in the genitalia, and those of the head, including antennal modifications and the arrangement and color of scales on the head and labial palps. Horak and Brown (1991) identified the characteristic large flat papillae anales (ovipositor lobes) of female tortricids as the single apomorphic trait linking the entire family. This was also described independently by Dugdale (1988) who incorporated this character into a key to superfamilies.

Prior to Horak and Brown's (1991) phylogeny, our understanding of tortricid relationships was mostly reliant on J.A Powell's (1964) phylogeny based on biology, and Kuznetsov and Stekolnikov's (1984) phylogeny, which was based on musculature of the male genitalia of Palearctic Tortricidae. Razowski (1976) also proposed a phylogeny based on Palearctic Tortricidae, however, like Kuznetsov and Stekolnikov's phylogeny, he did not identify which characters support the branches nor did this phylogeny include outgroups. Thus, symplesiomorphies and synapomorphies in these phylogenies were not differentiated, and the bulk of their taxa are defined by hypothesized shared losses (Regier et al., 2012). Although Razowski (1976) and Kuznetsov and Stekolnikov (1984) examined a limited range of characters, their phylogenies were based a broader range of taxa than Powell (1964). The phylogeny of Powell (1964) was restricted to North American species of Tortricinae and was based on a selected group of morphological and

biological characters; it differentiated olethreutines from tortricines based on the loss of internal feeding habits in non-olethreutines. Based on the world fauna, external feeding habits, including leaf rolling and leaf webbing, are found throughout the family, whereas, internal feeding as borers in roots, stems, and fruits is present in speciose genera of Eucosmini, e.g., Eucosma and Epiblema, and most Grapholitini (Horak & Brown, 1991)

Horak and Brown (1991) noted the lack of serious efforts towards a worldwide synthesis for the Tortricidae. Since then, there have been improvements to our understanding of phylogenetic relationships within the family. Horak (2006) resolved many relationships within the Olethreutinae of Australia. Relying on the phylogenetic distribution of pheromones in 530 species of tortricids, Safonkin (2007) proposed the first phylogeny to recognize three subfamilies within the family. Aside from a few minor proposed changes in tribal assignment, Endotheniini to Bactrini (Dang, 1990), and Gatesclarkeanini into Olethreutini (Horak, 2006),the classification of the Tortricidae has remained stable in the last twenty years. That being said, our current understanding of phylogenetic relationships is characterized as being “highly provisional” (Regier et al., 2012).

The results of my study will be an explanatory hypothesis in the form of: (1) a hand made phylogeny based off the tree topology found in Regier et al. (2012) with 52 non-traditional and traditional morphological characters mapped onto this phylogeny, and (2) a phylogeny based solely on the aforementioned morphological characters. I hypothesize that this approach will reveal valuable, novel, taxonomically informative morphological characters that can be used to delimit tribes and potentially predict evolutionary relationships.

The second chapter of this thesis concerns methodology that will be essential for this study. In order to examine morphological structures of the family Tortricidae, I evaluated the different stains used by currently practicing systematists to colorize and differentiate tissue constituents. The results from this evaluation are presented in the second chapter, complemented by a comprehensive investigation into staining factors that affect performance. Based upon this investigation, I determine which staining method is most appropriate for slide preparations in the final chapter.

In chapter III, I made whole-body dissections of de-scaled moths and slide mounted them following a technique described by Lee and Brown (2006) (see Methodology) and developed a character matrix for all 22 tribes within the Tortricidae. I assess the morphological variation within the Tortricidae using non-traditional characters of de-scaled adult specimens as well as some traditional characters, including those on the genitalia. This approach focuses on identification of novel structures on the exoskeleton and their utility in inferring phylogenetic relationships within the family. These data are used to infer phylogenetic relationships at the tribal and subfamily level.

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CHAPTER II

ANALYSIS OF HISTOLOGICAL STAINING TECHNIQUE OF LEPIDOPTERA

Introduction

Insect identification routinely involves dissection of genitalia to view diagnostic structures. Systematic research that involves characters of the male and female genitalia also require high quality dissections. Preparing specimens for dissection requires some form of tissue softening and clearing, often using a 10% solution of potassium hydroxide (KOH), followed by manual cleaning (often in ethanol) and staining. Unfortunately, most tissues do not retain their natural color after being processed, making their components difficult to view under a light-microscope (Presnell & Schreibman, 1997). The information lost by removing the natural color from tissues can be preserved through the process of staining, which allows for differentiation and identification of tissue components.

In Entomology, there is no widely accepted standardized procedure for specimen preparation, particularly with regards to dissection technique. However, within certain taxa (e.g., Lepidoptera), there are published protocols available for dissection and slide mounting (e.g., Lee & Brown, 2006). A consensus in the entomological community regarding staining standards is currently lacking. However, there are a limited number of resources available with staining information as it pertains to insects (e.g., Barbosa et al., 2015). Although there is a lack of effort to understand histological methods among

entomologists, it is highly monitored in the histopathology discipline to ensure diagnostic accuracy (Furness, 2002).

Histopathologists have variable concepts of the perfect stain, but have come to a general consensus in practice that has led to uniform and improved staining standards (Furness, 2002). For example, the features assessed in a standard H&E (hematoxylin & eosin) section include but are not limited to: hematoxylin intensity and color differentiation, eosin intensity, eosin selectivity, and uneven staining (Furness, 2002). Although these qualities do not go unnoticed in insect preparations, their optimization is poorly understood, and this leads to difficulty in trouble-shooting common staining problems (e.g., uneven staining, low intensity, poor tissue selectivity). Furthermore, achieving accurate identification and interpretation of a specimen preparation is encumbered, or impossible, if the stained tissue does not accurately reflect the true composition or arrangement of structures.

Horobin (2002d) used the term "affinity" to describe the tendency of a stain to transfer from the solution to tissue, i.e., to describe the forces that bind dye to tissue. Horobin (2002d) further describes four interactions that must be considered to maximize affinity; these are stain-tissue, solvent-solvent, stain-solvent, and stain-stain.

A review of biological staining and factors that affect stain quality

Prior to the middle of the nineteenth century, there was no competition for natural dyes such as cochineal, carmine, hematoxylin, saffron, indigo, and orcein (Presnell & Schreibman, 1997). Clark et al. (1983) suggested orcein is the first synthetic dye, as it was produced (named French purple) about 1300 A.D. by exposing orcinol, derived from

lichens, to air oxidation "in the presence of ammonia formed in fermented urine by urea; orcein's historical classification as a natural dye was perhaps due to the original sources being raw materials, however, orcinol has been produced industrially, for dye manufacture, since the 19th century (Horobin, 2002c).

In 1856, 18 year old William Perkin was challenged by his professor to synthesize quinine, a treatment for malaria; in doing so, Perkin accidentally created mauvine, "the first synthetic dyestuff to be manufactured from organic chemicals derived from coal tar" (Holme, 2006). Although regarded as the pioneer of the synthetic dye industry, Perkin's mauvine was not the first synthetic dye; Woulfe, in 1771, and Laurent, in 1842, both prepared picric acid through different means to dye silk bright yellow, and Runge, in 1834, prepared aurin from crude carbolic acid (Holme, 2006). A purple dye, murexide, was also made from the uric acid derived from bat guano in the 1850's by Robert Rumney Ardwick (Holme, 2006). What made Perkin's mauve significant, and often given credit for being the first synthetic dye, was it's highly desirable color, making it one of the most valued textile dyes of that time (Holme, 2006); because of this, Perkin patented his mauvine months after it's discovery. Since Perkin's synthesis of mauvine, natural dye production and use has gradually diminished due to the limitations of natural dyes including color yield, dyeing efficiency, cultivation efficiency, shade range, reproducibility, availability, effluent, and toxicity (Holme, 2006).

However, the exception to recent dominance of synthetic dyes is hematoxylin (Presnell & Schreibman, 1997; Titford, 2005). Extracted from the heartwood of *Caesalpina campechianum,* a legume native to Central and South America, hematoxylin was one of the first histological dyes and continues to be one of the most used and well

known natural dyes (Presnell & Schreibman, 1997). Although discovered in 1502 by Spanish explorers and traded vigorously throughout Europe for dyeing fabrics, it wasn't until the mid 1800's that histologists would use hematoxylin to stain cellular components (Titford, 2005). Initially, hematoxylin produced unsatisfactory staining because it was not colorfast; however, it was discovered by Frederick Böhmer (1865) that hematoxylin's maximum potential as a stain could be reached in the presence of a mordant, a metal salt that fixed the color (Horobin, 2002d).

In 1874 Polish chemist Heinrich Caro synthesized a yellow-red dye and called it eosin- after a nickname, Eos, of a girl he admired (and of the goddess of Dawn – Eos). One year later, the German chemist Emil Fischer published a paper on eosin Y, introducing it to the histological discipline (Fischer, 1875).

The first double-staining technique was introduced by Schwarz (1867), and nearly a decade later, hematoxylin was used in conjunction with eosin (H&E) for the first time by chemist Wissowzky (1876) – revolutionizing the histological discipline (Gill, 2012). H&E staining techniques were designed that are still implemented, primarily for paraffin embedded histological sections; this technique was not developed for whole tissue mounts such as a genitalia dissection. Pantin (1948) describes how to prepare a hematoxylin solution for staining whole mounts (taxon not specified) using absolute alcohol, acetic acid, glycerol, and water, however he does not indicate how well this stains tissue. Excluding the aforementioned author there is little evidence that hematoxylin has been used for whole tissue mounts.

Used for both histological sections and whole tissue mounts, eosin Y (eosin yellowish) has become widely used for dissections of Lepidoptera (e.g., Goodson et al.,

1993; Koster, 2010; Kristensen, 1984; Lee & Brown, 2006), although the related compound, eosin B (also known as eosin bluish), has been used by some researchers (Davis & De Prins, 2011); Kaila (1999) used "red eosin", however it is unclear if this is a synonym for eosin Y (Acid Red 87) or eosin B (Acid Red 91). When compared to other popular stains, safranin, chlorazol black E, orange G, and mercurochrome, specimens stained with eosin Y produced better images using confocal laser scanning microscopy, in part because of its higher levels of fluorescence (Lee et al., 2009). Few authors provided staining time required; in those that did, time varied greatly, ranging from "a few minutes" (Goodson et al., 1993) to ~4 hours (Lee & Brown, 2006). Stain concentration and solvent (alcohol vs aqueous) were not provided in any of the previously mentioned literature sources for eosin Y. Eosin's utility as a stain is highly dependent on the dye's preparation – unknown to many Lepidopterist's who use the dye. One of the most important variables that determines effectiveness of the eosin, and other "acid" dyes (anionic dyes), is pH (Gill, 2012; Presnell & Schreibman, 1997), which is described in detail in the "pH" section. The term "acid" dye, although used commonly, can be misleading as it implies the dye itself is acidic. Rather, "acid" dyes perform best when in acidic environments. "acid" dye is a simplified term for all the anionic dyes. Whereas "basic" dyes are all the cationic dyes. Other "acid" dyes commonly used in genitalic or whole tissue preparations include chlorazol black E, orange G, and acid fuchsin.

Since it's accidental discovery 80 years ago (Cannon, 1937), chlorazol black has become one of the most popular stains used by lepidopterists who regularly dissect genitalia (e.g., Adamski et al., 2014; Davis, 2012; Hodges & Adamski, 1997; Horak, 2006; Kaila, 1999; Kawahara & Rubinoff, 2012; Kristensen, 2015; Landry & Powell,

2001; Landry, 2007; Lee & Brown, 2006; Pantin, 1948; Pellmyr, 1999; Robinson, 1976; van Nieukerken, 1985). Landry (1995) has described chlorazol black as fixing "remarkably" well to insect cuticle, and having a high affinity for insect endocuticle, more so than exocuticle. Cannon (1937) described the dye as an "excellent stain for chitin which stains green, and probably for glycogen which stains pink or red". Its preparation varies, from using an aqueous solution (e.g., Landry, 1995; Zeller-Lukashort et al., 2007), a low concentration (<30%) alcohol solution (e.g., Landry, 2007), or a 70% alcohol solution (e.g., Horak, 2006; Pantin, 1948; Robinson, 1976). Many authors did not specify how their solution was prepared (e.g., Brown & Powell, 1991; Davis, 2012; Kristensen, 2015). Furthermore, very few authors report the concentration of chlorazol black they are using; some have stated that it is a "weak solution" (Brown & Powell, 1991) and others prepare a 1% or 2% solution (e.g., Davis, 2012; Horak, 2006; Robinson, 1976). A general consensus as to staining time for Lepidoptera preparations is that chlorazol black stains rapidly, requiring as little as 10 seconds (Lee & Brown, 2006); however, some have stained for several minutes (Landry, 1995; Pellmyr, 1999). Many are cautious when staining with chlorazol black due to the perceived difficulty of de-colorizing over-stained preparations (a simple method for de-staining over-stained preparations is provided below in the "decolorizing agents" section).

In 1878, sixty years prior to chlorazol black's synthesis, German chemist Heinrich Baum synthesized "Acid Orange 10", commonly recognized as Orange G, for the purpose of dyeing silk and wool (Ballard, 1991). Orange G's name is derived from it's colour when dissolved in water (acidified: yellow orange, orange; alkaline: orange brown) or ethanol (golden orange) and also for its synthesis that uses G acid (Ballard,

1991). In North America, Jean-François Landry pioneered the use of Orange G for genitalic mounts of Lepidoptera preparations, after following the suggestions of Italian entomologist, Giorgio Baldizzone, in a letter dated 18 May 1990 (Landry, Pers. comm., 2017). Since then several other North American taxonomists have adopted J-F Landry's technique (Kawahara & Rubinoff, 2012; Landry & Powell, 2001; Metzler, 2016). Landry first published on his use of Orange G in 1995 (Landry & Wagner, 1995) where he describes his preparations as being "stained with both Orange G (in 30% ethanol; enhances sclerites) and chlorazol black (in 70% ethanol; enhances membranes)". Neither J-F Landry, or subsequent authors who adopted his method, provide information regarding the concentration of orange G stain or how long specimens are typically stained. Although not common, according to North American literature, the use of lactic acid in Landry's orange G preparation is an effective way to neutralize remaining KOH in specimens (Landry, 2007). Bernard Landry, who worked as a support technician for J-F Landry, also adopted the use of orange G lactic acid (Landry, Pers. comm., 2017) and subsequently published on its use in several journal articles (e.g., Landry & Powell, 2001; Schmitz & Landry, 2005). The late Danish morphologist, Niels Kristensen, examined KOH-treated material in lactic acid (Kristensen, 1979). In the pH section, I discuss the importance of using an acid when working with anionic stains, not just for neutralizing KOH but for achieving high affinity stain solutions. Although the previously mention dyes (Chlorazol Black, eosin, Orange G) are anionic, their names do not suggest their acid nature, perhaps for this reason, few authors have used acidifying agents to prepare material for examination. Acid fuchsin is an exception to this, as the name implies, it is an acid/anionic stain.

In 1877, one year prior to orange G's synthesis, Heinrich Caro prepared acid fuchsin by sulfonation of basic fuchsine (three years after his discovery of eosin in 1874) (Horobin, 2002a). Acid fuchsin was initially used as a biological stain for blood smears, mitochondrial staining, and histopathology; however staining techniques using acid fuchsin have been applied to embryo sections, resin-embedded tissues, and whole organisms such as fungi (Horobin, 2002a). Although less commonly referenced in North American literature for preparing insect dissections (not sections), acid fuchsin has been used internationally for staining both wings and genitalia preparations (e.g., Davis & De Prins, 2011; De Benedictis & Powell, 1989; Hogue, 1963; Hood, 1953; Horak, 2006; Landry, 1995). Acid fuchsin has been prepared through acidification by various methods, using picric acid (De Benedictis & Powell, 1989), acetic acid (Horak, 2006; Landry, 1995), or lactic acid (Landry & Powell, 2001); perhaps because the name implies acidity, those who use it are more likely to prepare it in an acid solution – other anionic stains do not obviously state this. Due to acid fuchsin's popularity as a histological stain, a recipe, which includes acidification, can even be found on Wikipedia. Without having a histological background, many morphologists are left unaware that many dyes are either cationic or anionic (or amphoteric: exhibiting properties of both cationic and anionic stains, e.g., orcein), and that pH has implications for stain affinity – thus anionic and cationic dyes are also called "acid" and "basic" stains.

Seemingly less common in modern morphological studies, the cationic/basic dyes include Mercurochrome, one of the most historically popular dyes used by lepidopterists, (Adamski, 1989; Adamski et al., 2014; Busck, 1942; Clarke, 1941; Davis, 2012; Hodges & Adamski, 1997; Landry, 1991; Powell, 1964; Robinson, 1976). Mercurochrome's

history dates back to 1889, when Dr. Ira Remsen discovered phenolsulphonphthalein (phenol red) (Dunning, 1927). Dr. Remsen submitted his discovery to the pharmacological department at John Hopkins Medical School where it was found to be extremely valuable in estimating kidney function (Dunning, 1927). Mercurochrome's synthesis was born of the suggestion that a "valuable genito-urinary antiseptic" could result from combining phenolsulphonphthalein with an antiseptic metal such as mercury or silver (Dunning, 1927). The former was chosen, and in 1919 a preliminary report of experimental and clinical studies of the germicide "Mercurochrome -220" was described (Young et al., 1919). Not even a decade later, Carpenter and Gathercoal (1926) would publish on the histological uses of the germicide, including the "striking advantage" being that the color is not removed from starch or cellulose "even after prolonged washing with alcohol, oil of cloves, or water". Carpenter and Gathercoal (1926) make an important remark about using mercurochrome in double staining methods involving "acid" dyes: "Care should be exercised to avoid the use of "acid" dyes. It is very sensitive to acid, the mercurochrome being converted by even very dilute acids to a yellow resinouse substance. Objects stained with mercurochrome change to a light yellow color when treated with an acid". It is only by luck that the lepidopterologists that use mercurochrome in conjunction with chlorazol black (Adamski, 1989; Landry, 1991) would have never discovered this because they did not acidify their chlorazol black stain. As with all previously mentioned dyes, many authors did not describe the solvent used to make the stain solution, however Landry (1991) states that he uses a 30% EtOH solution, where as Clarke (1941), Busck (1942), and Robinson (1976) used aqueous solutions. Prominent American Lepidopterologist John Franclemont also used mercurochrome,

however he never published his methods (Brown, Pers. comm., 2016). Staining time ranges from “a few minutes” (Powell, 1964), “10 minutes” (Lee & Brown, 2006), to “until stained to suit” (Clarke, 1941).

A final dye worth mentioning is safranine O, a dye structurally related to Perkin’s mauve. In 1859, Three years after the development of Perkin’s mauve, English scientist Charles Greville Williams discovered the azine dyes known as safranines. Although less common in North American literature, safranin O (Synonym: Safranin T), has been used to stain wings (Davis & De Prins, 2011), genitalia (Hardwick, 1950) and whole body mounts (Lee & Brown, 2006) of Lepidoptera. Phenosafranine, a closely related dye, has been used by Erik van Nieukerken (van Nieukerken et al., 2010) for staining genitalia of nepticulid moths; A personal observation, smaller moths, such as the Nepticulidae, have much smaller genitalia and often take longer to stain than larger, more sclerotized genitalia.

Frank Nelson Pierce, author of five books illustrating male and female genitalia of British Noctuidae (Pierce, 1909), Geometridae (Pierce, 1914), Tortricidae (Pierce & Metcalfe, 1922), and Tineidae (Pierce & Metcalfe, 1935), used two cationic/basic stains, carbol fuchsin, and carbol methyl blue. It would be valuable to re-examine Pierce’s preparations to determine how well the stains have preserved since 1909. Pierce was one of the first Lepidoptera taxonomists to describe his methods, particularly with staining, in great detail. Relative to other prominent taxonomists of the time, such as Busck, Heinrich, Burgess, Scudder, and Dampf, Pierce’s methods are very thorough. In his Noctuid book (Pierce, 1909) shared a sentiment that I agree with: “The examination of these parts is most interesting and often at times exciting. The marvelous variety and the

extraordinary beauty of the minutest parts, far surpassing many of the more popular microscopal studies, especially when a little stain is used".

All of the dyes previously mentioned, except hematoxylin, are synthetic dyes and can be used both progressively (leave in dye until correct stain is achieved) or regressively (purposefully over-stain, then de-colorize in solution to obtain correct stain). Presnell and Schreibman (1997) suggested the real importance of synthetic dyes is in their ability to double or triple stain the same specimen (use different dyes on the same preparation). As a result of their chemical structure, if chosen correctly, these dyes can stain tissue selectively because of known specificities to particular tissues or cell organelles (Presnell & Schreibman, 1997). Even very small differences in dye content, chemical structure, and presence of impurities can result in failed or inconsistent coloration of the specimen – this can lead to misinterpretations or misidentifications of microscopic structures (Biological Stain Commission website, 2017) . Historically, the textile dye industry was the only source of dyes for histologists, making it difficult to consistently obtain reliable dyes (Presnell & Schreibman, 1997). Although staining is credited as being brought into general use in 1858 by J. von Gerlach, who stained cellular components using carmine (extracted from cochineal insects), it's standardization did not occur for nearly a century (Presnell & Schreibman, 1997). At the time, batches of dye varied in their color and quality and also contained varying degrees of impurities. George Grübler, a German pharmacist and graduate student at the time, attempted to standardize dyes by obtaining batches from other firms and testing them for technical use. This was done in addition to his manufacturing of biological stains and selling them to scientists around the world; resulting in the name Grübler being "synonomous with fine stains"

(Presnell & Schreibman, 1997). The World Wars terminated the sale of Grübler dyes to England and America; this resulted in the formation of the Biological Stain Commission by H. J. Conn and his associates, in the early 1920s (Presnell & Schreibman, 1997; The Biological Stain Commission website). The commission worked with manufactures to test their products and "permitting approved batches to be put on the market with the stamp of the commission on them" (Presnell & Schreibman, 1997). Approved batches receive a a CI (Colour Index) number, which indicates that a sample was submitted to the commission, it proved to be true to type based on spectrophotometric testing, and had satisfactory dye content which was correctly indicated on the label. For example, eosin Y is CI 45380, and if this Colour Index number is not provided, then it either was not tested by the commission, or it failed to meet specifications for CI 45380 and is therefore not standard eosin Y. The Biological Stain Commission arranged a conference in 1974 where it was concluded that dyes are still not "fine chemicals but usually mixtures of variable and often undefined composition" (Lyon, 2002). Dyes have certainly improved since then, however dyes obtained prior to 1974 likely were tested with different expectations and may not perform as well as those produced later.

As previously mentioned, most literature is lacking detailed descriptions of stain preparation because many authors are unaware of the important factors that determine stain affinity. Presnell and Schreibman (1997) describe pH as being one of the most important factors of staining properties, whether it is aqueous or alcoholic. In the following sections, I describe how these factors can inhibit or strengthen staining.

The information provided in the below sections is based primarily from a histological perspective. Because this paper is intended to guide taxonomists in their use

of stains for whole preparations, this is important to note as results may vary when these practices are applied to whole tissue preparations.

pH

Although crucial to the staining process, pH of the stain has been overlooked and not provided by many authors. Outside of the histological discipline, no taxonomist has ever tested, or described, a method for preparing pH-correct stains for use in whole tissue mounts. Although overlooked, some who have used anionic stains have, coincidentally, acidified their stains, but for the purpose of neutralizing KOH (caustic potash used to soften/clear specimens). Pierce (1914) may have been one of the first to employ this method where he noted a permanent stain was difficult to obtain due to "the imperfect removal of the caustic potash"; to mitigate this problem, Pierce employed a washing process using citric acid, resulting in a "very satisfactory stain". Landry (2007) ensures remnant KOH is neutralized during the staining process by dissolving orange G in lactic acid and 30% ethanol. This method was "succinctly described" to J.-F Landry by Giorgio Baldizzone in a letter dated 18 May 1990; Baldizzone learned the technique from his mentor, and amateur German microlepidopterist, Eberhard Jäckh (Landry, Pers. comm., 2017).

Synthetic dyes can be either anionic (acid), cationic (basic), or amphoteric (cationic below a certain pH, the isoelectric point, and anionic above it) (Baker, 1958). This type of stain-tissue interaction is the most widely discussed and is termed electrostatic or coulombic interactions; this type of attraction arises from electrostatic attractions between unlike ions (Horobin, 2002d). "Basic" dyes are rich in coloured

cations, which are attracted to tissues constituents concentrated with anions (e.g., DNA, chromatin, RNA, mucous, lipids); acidic dyes are rich in coloured anions, which are attracted to tissues constituents concentrated with cations (e.g., collagen and cytoplasm) (Baker, 1958; Horobin, 2002d). A detailed account of mechanisms behind acid and"basic"dyeing is provided in Chapter 10 of Baker (1958). Because of the nature of amphoteric dyes (e.g., orcein), their affinity for tissue will differ depending on the composition of tissues (which can also be amphoteric, not just anionic or cationic), which could require the same dye to be used in both an acidic and a basic solution. Because of this, amphoteric dyes can be difficult to use with consistent results.

Baker (1958) explains how "acid" dyes will colour particular tissue constituents in strongly acid solutions, but will scarcely act above a certain pH. "Basic" dyes will act strongly on the same tissue constituent if in an alkaline solution. The affinity between the dye and tissue would fall dramatically and nearly disappear at about the pH as that at which the "acid" dye would failed to act – this is where the isoelectric point is estimated to exist (Baker, 1958) and there is no dye-binding at the isoelectric point (Presnell & Schreibman, 1997). Below the isoelectric point, proteins behave as bases, therefore binding to anions such as the "acid" dyes eosin Y, Orange G, and Chlorazol Black E (Presnell & Schreibman, 1997). The reverse is true above the isoelectric point. As a dye solution becomes more acidic, increasing amounts of "acid" dye will be bound. If the dye solution is alkaline, increasing amounts of "basic" dye will bind. Without knowing the pH of the dye solution, it is impossible to guarantee consistent dye binding. Manipulating the pH of a stain solution can aid in the enhancement or removal of anionic and cationic dyes (Presnell & Schreibman, 1997) (see Decolorizing section for information on

removing dyes in over-stained samples). Because of this, anionic ("acid" dyes) should be appropriately acidified in order to achieve consistent stain affinity (I use pH 4.5 for eosin Y). I have noticed with whole dissections that anything lower than a pH of 4.4 can result in "flat" staining, completely homogenized with no tissue differentiation (i.e. everything stains the same shade of flat pink/red). Kiernan (2014) states that, because eosin is not a "typical" "acid" dye, it should not be made too acidified or else "insoluble unionized eosin will be precipitate, leaving a colorless solutions"; I have witnessed this phenomena with eosin, but more so with Chlorazol Black E which can not be acidified as much as eosin (pH 4.5) before precipitating. Anything above a pH of 7.5 will inhibit eosin staining, greatly limiting stain affinity (Llewellan, 2008). Llewellan (2008) also states that adding acetic acid to eosin can result in brighter, but more homogenous non-specific staining; it is unclear if Llewellan is singling acetic acid out, or if this applies to all acids. In the following paragraph, Llewellan states that:

> *"Often the intent is simply to maximise the depth of colour with an acid dye, to cause the maximum dye to attach. This is much simpler, and just requires that an acid is added in sufficient quantity to bring the pH to about 3-4. Most commonly this is obtained with a 1% or 2% solution of acetic acid. That particular pH is important as tissue amino groups are most reactive at that pH. It is the tissue amino groups that bind acid dyes, remember, so the more of those that are able to react, the better. Of course, sufficient time and dye concentration have to be allowed for the attachment to take place fully"*

Ultimately, the affinity of a dye for tissue will depend on the structural macromolecules of the tissue constituents. These macromolecules have side chains that form either a positive or a negative charge (Kiernan, 2014). As mentioned earlier, positive ions are mainly associated with proteins. Since "acid" dyes are attracted to positively charges sites in tissue, they will bind well to the side chain of the amino acid arginine, for example, because it is a strong base – meaning it carries a positive charge even at a high pH (Kiernan, 2014). The chemical structure of the dye and that of the tissue constituents determine the electrical interaction that will occur between the two, and whether or not the dye will bind to the tissue and do so selectively (Horobin, 2002b) – by adjusting the pH of the stain solution, the user can tip the scale in favor of any dye, regardless of it's electrical nature (anionic or cationic). However, it is logical to use an anionic dye, in an appropriately acidified solution, and vice versa for a cationic dye. Finally, selectivity (differential tissue staining) is also dependent on how long a specimen is dyed – prolonging the staining process can cause basophilic materials to stain, even in acidified anionic stain solutions(Horobin, 2002d).

Acetic acid is used for dye acidifying for several reasons, including it's ease of availability and low cost. Acetic acid is common in most laboratories and is one of the oldest fixatives on record (Presnell & Schreibman, 1997). The allure of acetic acid as a fixative relies on it's ability to fix tissue without hardening it (Baker, 1958; Presnell & Schreibman, 1997) even after subsequent soaking in 80% alcohol, tissues remain extremely soft (Baker, 1958). Presnell and Schreibman (1997) state that acetic acid actually prevents some "hardening that, without it, might be induced by subsequent alcohol treatment". Another valuable property of acetic acid is it's swelling effect on

tissue, which can counteract some of the shrinkage caused by most fixing agents, (Presnell & Schreibman, 1997); this property is not unique to acetic acid, rather it is common to most acids because they break down cross linkages between proteins, and they release lyophilic radicals that associate with water molecules (Presnell & Schreibman, 1997). Ethanol causes serious tissue shrinkage and hardening (Presnell & Schreibman, 1997), however, staining with acidified dyes (using acetic acid), can both prevent shrinkage and slow the hardening effects of subsequent washes in alcohol. J-F Landry stopped using acetic acid, and switch to lactic acid to neutralize KOH, because "being a strong acid, it caused a lot of bubbles inside parts, and these could be difficult to dissipate" (Landry). Lactic acid is also more affordable than glacial acetic acid and might explain it's use in other laboratories.

Solubility

To determine the solubility of your dye, refer to StainsFile (Llewellan, 2012) or a histology textbook, such as *Conn's Biological Stains 10th edition* or *Humason's Animal Tissue Techniques 5th edition* which are referenced many times in this paper. Furthermore, solubility information for six common dyes (Chlorazol Black E, eosin Y, Acid Fuchsin, Safranin, Orcein, and Orange G) are provided in TABLE 2.1. It is important to know that many dyes are not soluble in commonly used dissection reagents (xylene, ethanol, isopropanol, water) and this will determine how much dye is available, in solution, to bind to tissue. I make 1% solutions for all my dyes, therefore they are all at least 1% soluble in the solution I prepare them in (typically ethanol). Substituting solvents, such as isopropanol in place of ethanol, is not wise, as many stains are not

soluble in isopropanol (Presnell & Schreibman, 1997). Because of this, it would also not be recommended to place stained specimens in water, as many stains are highly soluble in water and will therefore leach. Hood (1953) realized this and would refrain from placing acid fuchsin stained preparations in water, to prevent washing the stain out. Fortunately for Hood, acid fuchsin is also insoluble in xylene, because his preparations were placed in xylene before mounted in balsam. Eosin Y is highly soluble in water (44%) (Table 2.1) and therefore specimens stained with it should subsequently only be placed in >95% ethanol for dehydration, or 70% ethanol for brief cleaning before dehydration. Fortunately, whole tissue stained with eosin that is acidified with acetic acid will not harden quickly in 95% ethanol and can therefore be cleaned and manipulated safely for a short period of time before dehydrating. Hogue (1963) indicates that non-aqueous stains may be applied after a specimen has been dehydrated; for example, a dehydrated specimen could be placed in acidified eosin Y, as opposed to staining prior to dehydration. This is especially important for preparations that may have been under-stained or that decolorized slightly during the dehydration process. As a cytoplasmic stain, eosin's high water solubility can be used to facilitate differentiation of the staining through subsequent tap water washes, with further differentiation occurring during the dehydration process (Wilson & Gamble, 2002).

A problem that many morphologists may encounter, particularly those who use aqueous stains and dissect or clean specimens in alcohol, is blotchy, or imprecise differential tissue staining. Alcohol dehydrates tissue, making it hydrophobic. If specimens are cleaned in alcohol solutions, followed by staining in an aqueous solution, the stains lack optimal affinity for the tissue that repels the dye-attached water molecules.

Because most stains are soluble in alcohol, it is advisable to use ethanolic stain solutions to prevent this from occurring.

Temperature

Temperature can play a role in stain affinity, however because no taxonomist has ever indicated if they heat or cool their dye while staining a specimen, I will not go into details on how temperature can affect staining. However, Baker (1958) indicates that high temperatures increase the rate at which dye-ions diffuse and also reduces their tendency to aggregate into larger particles; whereas cold temperatures encourage dye ions to aggregate into large particles. If dye ions are aggregated in large groups, it makes it less likely they will attach to oppositely charged groups of proteins (i.e. tissue components) because they move slower and penetrate less easily (Baker, 1958). Although higher temperatures might encourage faster stain uptake, it does not increase the amount of stain that can eventually be taken up by the tissue. Using wool as an example, Baker (1958) describes how 1 hour in dye at 100°C may suffice for wool to take up as much dye as it can hold, if the temperature were reduced to 20°C, it might require 5 months to elapse before the same dye can reach equilibrium with wool. Nearly half a year may seem extravagant, however, Baker states that "most biological material is very much more easily penetrated by dyes than wool, and the temperature is only raised above that of a laboratory when a dye has a special tendency to flocculation or when a tissue-constituent is particularly close-textured and therefore difficult to penetrate (e.g.mitochondria)".

Water quality

Because tap water contains alkalis, stains sensitive to alkalis will leach, such as Acid Fuchsin, eosin Y, and Orange G. Tap water can actually be used to decolorize over-stained samples (See Decolorizing agents section) (Hogue, 1963; Hood, 1953). Tap water varies in quality depending on the source and quality control measures, therefore the hardness (or presence of alkalines) will vary throughout the year and may yield inconsistent results if used as a carrier for stain. Distilled, purified water near pH neutral is ideal for stain preparation.

Decolorizing agents

As mentioned earlier, Hood (1953) would avoid placing stained preparations in water due to acid fuchsin being highly soluble in water. In order to decolorize acid fuchsin stained specimens, he would place them in tap water. Matthews (1998), inventor of the CSIRO vesica everter, used 30% alcohol to de-colorize specimens that were over-stained with orcein. Because Matthew's preparations are dissected in isopropanol, and stained in an absolute isopropanol orcein solution, the water works quickly to rehydrate and remove stain from tissue. Previously, it was thought that once overstained with Chlorazol Black, it was extremely difficult to reduce the degree of coloration (Robinson, 1976), however I have found that Windex® will easily de-colorize overstained samples within seconds. Windex® was chosen because I suspected a strong base would aid in removing "acid" dyes from stained tissue; upon realizing this, the nearest basic solution on my desk was Windex®. I immersed over-stained specimen in Windex® for 10 seconds, rinsed (70% ethanol), then viewed under a microscope to determine if another

treatment is needed. This can also be used as a method of regressive staining, where specimens are purposely over-stained and then de-colorized to obtain ideal tissue differentiation (Baker, 1958; Presnell & Schreibman, 1997). Landry (1995) came to the same conclusion, although nearly twenty years before me, in which a strong basic solution will de-colorize stained preparations; in his case, those stained with chlorazol black could be de-colourized with hydrogen peroxide of sodium hypochloride (Javex®). Recall that chlorazol black is an anionic dye; cationic dyes (e.g., mercurochrome) are not easily removed from tissue using basic solvents, rather they compete with the dye for the anionic components of the tissue. Baker (1958) describes this and shows that acidified alcohol will rapidly remove colouring of tissue by a cationic dye, and will leave intact the colouring by anionic dyes. Presnell and Schreibman (1997) further corroborate this and state that acid solutions are used to remove excess "basic" dyes, and alkaline solutions remove excess "acid" dyes. Finally, cationic (basic) dyes (e.g., mercurochrome, safranin O) are more resistant (than anionic) to washing in water, however are quickly removed by alcohols used in dehydration, which removes anionic dyes much more slowly. Dehydrating alcohols act like weak acids, their ability to extract cationic dyes "diminish in the series methanol, ethanol, propanol, butanol, pentanol" (Baker, 1958). Because of this, the use of an anionic dye may be preferable if dissections are carried out primarily in alcohol.

Testing stain performance

A small-scale test of nine stains simulated the rigorous investigation of staining previously discussed. After learning about the importance of dye solubility on StainsFile

(Llewellan, 2012), I wanted to determine which of the nine stains, in both aqueous and ethanolic solutions, would be appropriate for whole mounts of the exoskeleton of Lepidoptera, specifically the male genitalia and wing venation. The purpose of this was to optimize staining techniques, identify and solve common staining issues, and achieve better stain performance in less time. The identification and resolution of common staining problems has been addressed in the previous sections (pH, and solubility particularly). Although many authors do not publish how long they stain preparations, others provide times that vary greatly with some staining overnight (Brown, 2016; Hogue, 1963; Landry, 1995), or for a few minutes to an hour (Goodson et al., 1993; Hardwick, 1950; Lee & Brown, 2006; Matthews, 1998; Powell, 1964). Because I wanted to identify a stain that could perform well in a short amount of time, I tested aqueous and ethanolic stain solutions at both 45 and 90 minutes for wing dissections, and 45 minutes for genitalia (due to limited availability of genitalia from the same species). The stains were tested on wings and male genitalia of *Eucosma awemeana* all collected during the month of June, 2015 in Starkville, Mississippi. Specimens were killed used cyanide and stored for a maximum of 3 weeks before being dissected.

Methodology

The nine stains tested were safranin O, mercurochrome, rose bengal, orange G, eosin Y, chlorazol black E, lignin pink, acid fuchsin, and double stain (a pre-mixed solution of acid fuchsin, lignin pink, GAA, lactic acid, and phenol available from BioQuip®). The former three being “basic” or cationic dyes, and the latter six being “acid” or anionic dyes. Double stain tested as having a pH of 2.0 and is composed of

"acid" dyes. Although nine stains were tested, one of them, eosin Y was also tested after being acidified with acetic acid, to a pH of 4.5; acidified eosin Y was only tested in the ethanolic form. Both aqueous (Millipore water) and ethanolic (80% EtOH) solutions were prepared for each dye (excluding the pre mixed double stain, and the aforementioned acidified eosin Y which was only prepared in ethanol). Ethanolic eosin Y was acidified because the author uses ethanolic eosin Y and wanted to determine if acidifying the solution improved staining (as was suggested by initial investigations). This resulted in 18 solutions being tested (8 aqueous, 9 ethanolic, and the pre-mixed double stain). Stains were prepared in a saturated 2% weight/volume solution, with the exception of the double stain, and chlorazol black which was prepared as a 1% solution because of it's highly concentrated dye content and ability to stain tissue very quickly. All non-absolute alcohol used was mixed with purified Millipore water only. See the decolorizing agents section regarding tap water and why it is not desirable in stain solutions.

Wing venation slides were prepared as follows. The forewings were removed and placed in a watch glass with 20% EtOH and a drop of bleach which acts to clear the scales. Kolinsky sable hair brushes were used to carefully denude the wing. If scale removal was difficult, the wing was moved to a 50% solution as scales were easier to remove as the EtOH concentration increased – although the wing membrane became more fragile and was susceptible to tearing. Once cleaned, the left and right wings were rinsed in 100% EtOH or Millipore water; this was dependent on which stain was used – for example, specimens to be stained in aqueous solutions were rinsed with water. After rinsing, the left and ring wings were placed in the same well in a 24-well plate, and stained with the same stain solution (either aqueous of ethanolic), however, one would be

removed at 45 minutes, and the other would get a full 90 minutes in the stain; wings stained with chlorazol black were only stained for 1-minute due to this stain's fast acting nature. Falcon™ Polystyrene 12- or 24-well plates were used because of their design for ease of maintaining sample ID's associated with each well, and unlike many glass or porcelain dishes, their flat-bottom, cylindrical wells deterred the dispersal of the stain up the side of the well. After being stained, wings were dehydrated in absolute alcohol for up to an hour followed by mounting in Euparal.

Abdomens were placed in 10% KOH overnight at room temperature. The following day they were initially cleaned in KOH using 000 synthetic hair paintbrushes (camel's hair or sable hair is organic material and can be digested by the KOH). Both synthetic and sable brushes were used subsequently to clean the abdomens in EtOH in series from 50%, 70% and finally 100%. Genitalia were removed from the abdomen usually in the 70% EtOH and cleaned separately. Both the abdomen and genitalia were placed in one of the 18 stain solutions for 45 minutes. After staining, the specimens were dehydrated overnight in absolute alcohol with glass chips covering them in specific positions. The following day they were mounted in Euparal.

Images of all preparations were taken using a Leica DFC 495 digital camera mounted on a Leica Z16 Microscope with motorized z-stepping, and image stacks were merged using Leica Application Suite V 4.1.0 with Montage Module.

Results

Figures 2.1-2.4 show all stained wing preparations. Except for ethanolic safranin, ethanolic acified eosin Y, and double stain, most stains did not sufficiently stain the

wings in 45 minutes. Eosin Y stained the wing sufficiently in 45 minutes, however it appeared blotchy. 90 minutes was sufficient for some of the dyes to achieve satisfactory stains, however many did not stain wing veins completely, or appeared patchy. Several stain solutions overstained the wings in 90 minutes, such as aqueous acid fuchsin, ethanolic safranin, aqueous safranin, and double stain. Aqueous mercurochrome may be overstained to view some structures, more importantly; it did not completely stain several wings veins (e.g., A1+2) completely. Chlorazol black did not stain wings sufficiently at all. Acidified eosin Y stained wing preparations best, at both 45 and 90 minutes; relative to the other preparations, the membranous areas stained an even shade of pink, while the veins mostly stained darker pink/red making it easy to differentiate between a vein and membrane. Ethanolic orange G, and ethanolic acid fuchsin also stained well at 90 minutes, however these failed to stain at 45 minutes.

Figures 2.5-2.6 show abdomens stained for 45 minutes. Many of the dyes stained satisfactorily, particularly those that differentially stained sclerotized areas and inter-segmental membranes; these include ethanolic eosin Y, ethanolic and aqueous mercurochrome, ethanolic and aqueous safranin, ethanolic acidified eosin Y, and double stain. Ethanolic and aqueous chlorazol black sufficiently stained the tissue, however there was no tissue differentiation; rather it appeared as a flat homogenous stain. Orange G, and rose bengal failed to stain sufficiently in 45 minutes. Ethanolic and aqueous lignin pink did stain abdomens sufficiently, however it was faint and therefore not satisfactory, as most preparations fade slightly over time.

Figures 2.7-2.8 show stained male genitalia. Aqueous acid fuschin, ethanolic safrinin, and double stain over-stained the tissue in 45 minutes. Aqueous mercurochrome

stained evenly, however it could be considered over-stained by some morphologists. Aqueous chlorazol black over-stained the left half of the preparation, but failed to stain the right half completely. Ethanolic orange G stained satisfactorily, however it is difficult to tell if it was stained at all, because the dye is similar in colour to the tissue colour. By comparing this preparation to others, it is clear that it is darker than those that did not stain well (e.g., Aqueous chlorazol black). Acidified eosin Y, and ethanolic and aqueous eosin Y stained satisfactorily. Several dyes failed to stain the tissue satisfactorily and appear faint or unstained; those include ethanolic lignin pink, ethanolic and aqueous rose bengal, ethanolic chlorazol black, and aqueous orange G. Acidified EO out-performed EO in all preparations

Discussion and Recommendations

After investigating the mechanisms behind staining and troubleshooting why several of the stains did not perform well, it was obvious that many of the solutions were not prepared to optimize their staining affinity. This is particularly true for the acid stains (acid fuschin, eosin Y, orange G, rose bengal, and chlorazol black) which could have performed better if they had been acidified.

All material was dissected in ethanol and subsequently dehydrated in absolute alcohol either briefly (wings), or overnight (abdomens/genitalia), which can cause leaching of aqueous and 'basic' stains (Baker, 1958; see decolorizing section). Because wings were not dehydrated in alcohol over night, this may explain why several of the aqueous stains that performed well in wings failed to achieve better stain affinity than their ethanolic forms in abdomens and genitalia. Aqueous eosin Y performed much better

in wings (Fig. 2.1 G-H) relative to the abdomens (Fig. 2.5 D) and genitalia (Fig. 2.7 D). Other stains performed better in aqueous solutions for abdomens and genitalia, such as acid fuchsin (Fig. 2.5B and Fig 2.7 B); although the genitalia in Fig. 2.7 B are over-stained, it is obvious that there was high stain affinity, but perhaps less time is needed to achieve optimal staining. Finally, cleaning in ethanol, then staining with an aqueous stain may have contributed to poor stain performance because dehydrated tissue becomes hydrophobic making it difficult for aqueous stains to penetrate the tissue in a short amount of time.

For over-stained preparations, a regressive staining method could have been used or less time in the solution may have resulted in optimal staining (See decolorozing section). Furthermore, the author does not recommend making 2% solutions as a 1% solution will stain equally well and it will conserve the dye. I used 2% solutions to ensure that dye concentration was at a level high enough that any issues with staining couldn't be attributed to low concentration. Most histologists use 1% or less. Perhaps the high concentration of dye also contributed to over-stained preparations.

Preparations being softened/cleared in KOH and wings being cleaned in a bleach solution, could have an increase in the pH of the tissue ultimately affecting what dyes can attach easily. Neutralizing remnant bleach or KOH by placing specimens in lactic acid, or acidified alcohol (EtOH + glacial acetic acid) could have controlled for this. Furthermore, the pH of the alcohol used to prepare dye solutions was not tested and this may have contributed to the surprising results of alcoholic eosin Y failing to perform as well as aqueous eosin Y in all preparations. As discussed in the pH section, eosin Y can not effectively bind to tissue above pH 7.5. At the time of this experiment, the importance of

pH was just being investigated and the author became aware of eosin Y's acid nature, as well as other acid stains shortly after.

Conclusion

After investigating the importance of pH, I am confident that many of the stains used in this study could perform well if prepared at the appropriate pH for their dye nature. Furthermore, because many stains are soluble in ethanol and perform well in ethanol, it is logical to mix stain solutions in ethanol if dissections and dehydration are to be done using alcohol. This avoids any negative interactions that may occur between alcohol, water, and tissue. Future experiments used to test stain affinity should consider pH of the stain, dissecting solution (usually alcohol), and the tissue (tissue pH may change after KOH or bleach treatment). Finally, females were not tested in this study, and the results may be different for females that have more membranous tissue – for example, chlorazol black performs well with female genitalia, however it did not stain sclerotized male genitalia as well. The variable results show a need for understanding mechanisms of staining and how to manipulate those to optimize staining and achieve consistent results.

Table 2.1 Characteristics of popular histological dyes

Name and Colour Index	Dye Classification	Acid or Base	Solubility (%)			Stability: light fastness	Reference page
			Water	Ethanol	Xylene		
Chlorazol Black (30235)	Anionic Polyazo	A	6	0.1	insoluble	Good	140
Eosin Y (45380)	Anionic Hydroxyxanthene	A	44	2	--	Good	231
Acid Fuchsin (42685)	Anionic Triarylmethane	A	10-12.5	0.1-0.3	insoluble	Poor	175
Safranin (50240)	Cationic Azine	B	4.5	3.5	--	Poor	274
Orcein (1242)	Oxazines and related dyes	A/B	0-2	0.1-4	--	--	290
Orange G (16230)	Anionic Monazo	A	10.9	0.2	insoluble	Moderate	113

Notes: Eosin Y, the sodium salt, is listed, not to be confused with the less common “free acid” eosin Y (C.I. 45380:2) which is also included in Horobin & Kiernan (2002). Orcein is amphoteric, thusly indicated as being both an acid and a “basic”stain. Solubility information may differ from other literature as higher and lower solubilities have been reported and are acknowledged in Horobin & Kiernan (2002). Solubility in xylene is not available for all dyes. Reference page refers to which page in Horobin & Kiernan (2002) data was extracted from

Figure 2.1 Wings stained with acid fuchsin and eosin Y.

Notes: Wings stained for 45 minutes (left) and 90 minutes (right). A-B, ethanolic acid fuchsin. C-D, aqueous acid fuchsin. E-F, ethanolic eosin Y. G-H, aqueous eosin Y.

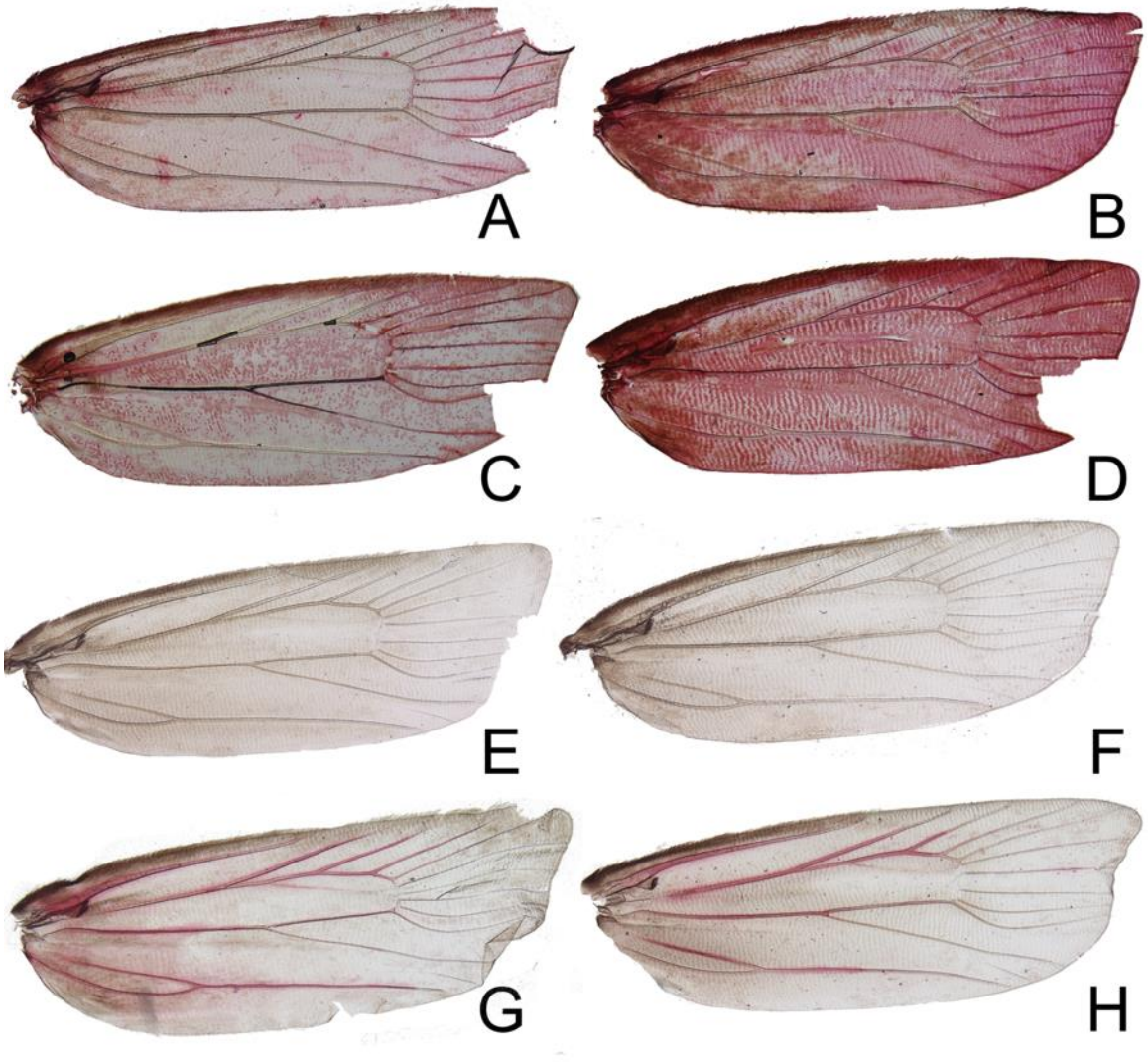

Figure 2.2 Wings stained with acid fuchsin and eosin Y.

Notes: Wings stained for 45 minutes (left) and 90 minutes (right). A-B, ethanolic acid fuchsin. C-D, aqueous acid fuchsin. E-F, ethanolic eosin Y. G-H, aqueous eosin Y.

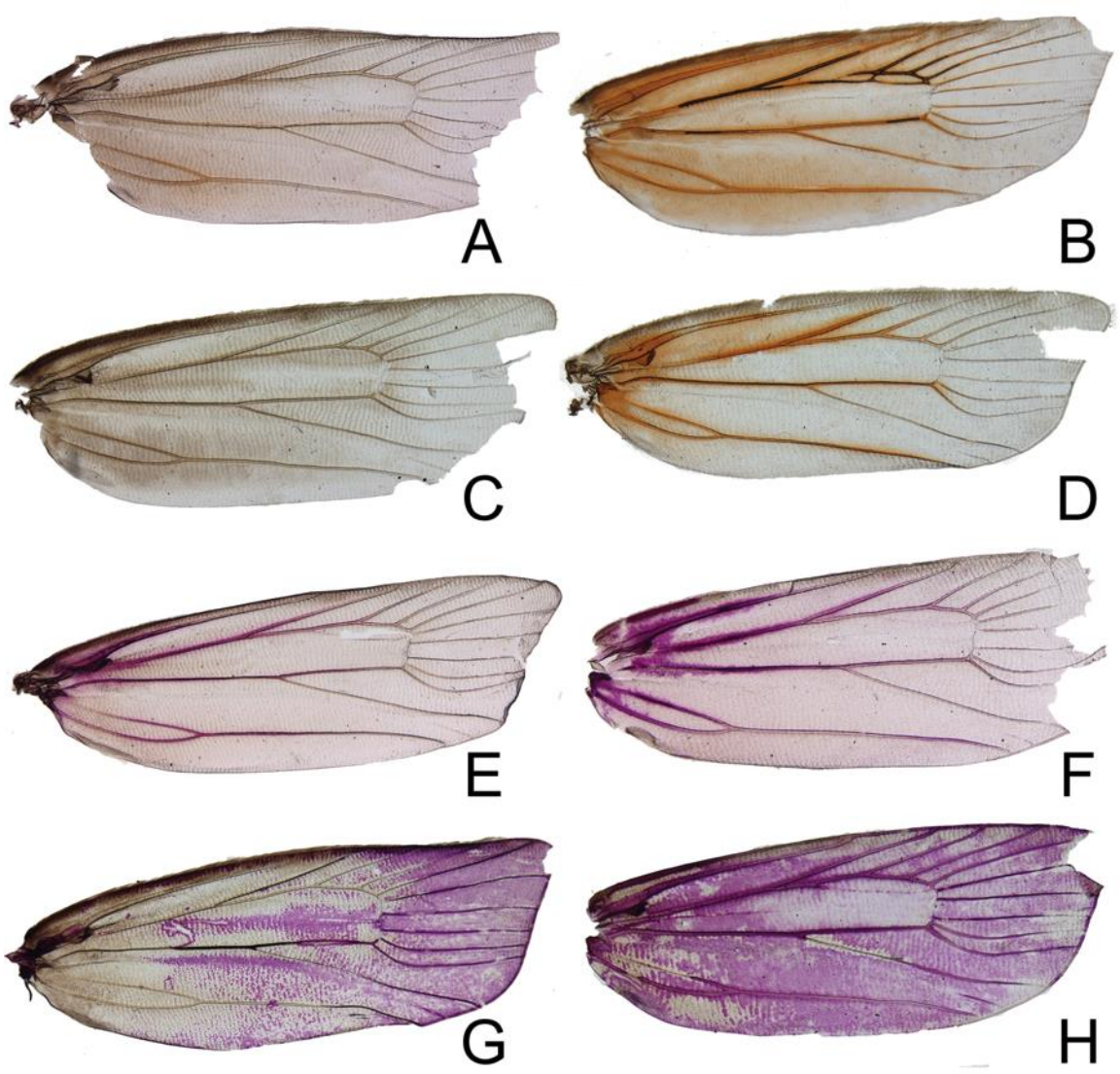

Figure 2.3 Wings stained with orange G and rose bengal.

Notes: Wings stained for 45 minutes (left) and 90 minutes (right). A-B, ethanolic Orange G. C-D, aqueous Orange G. E-F, ethanolic rose bengal. G-H, aqueous rose bengal.

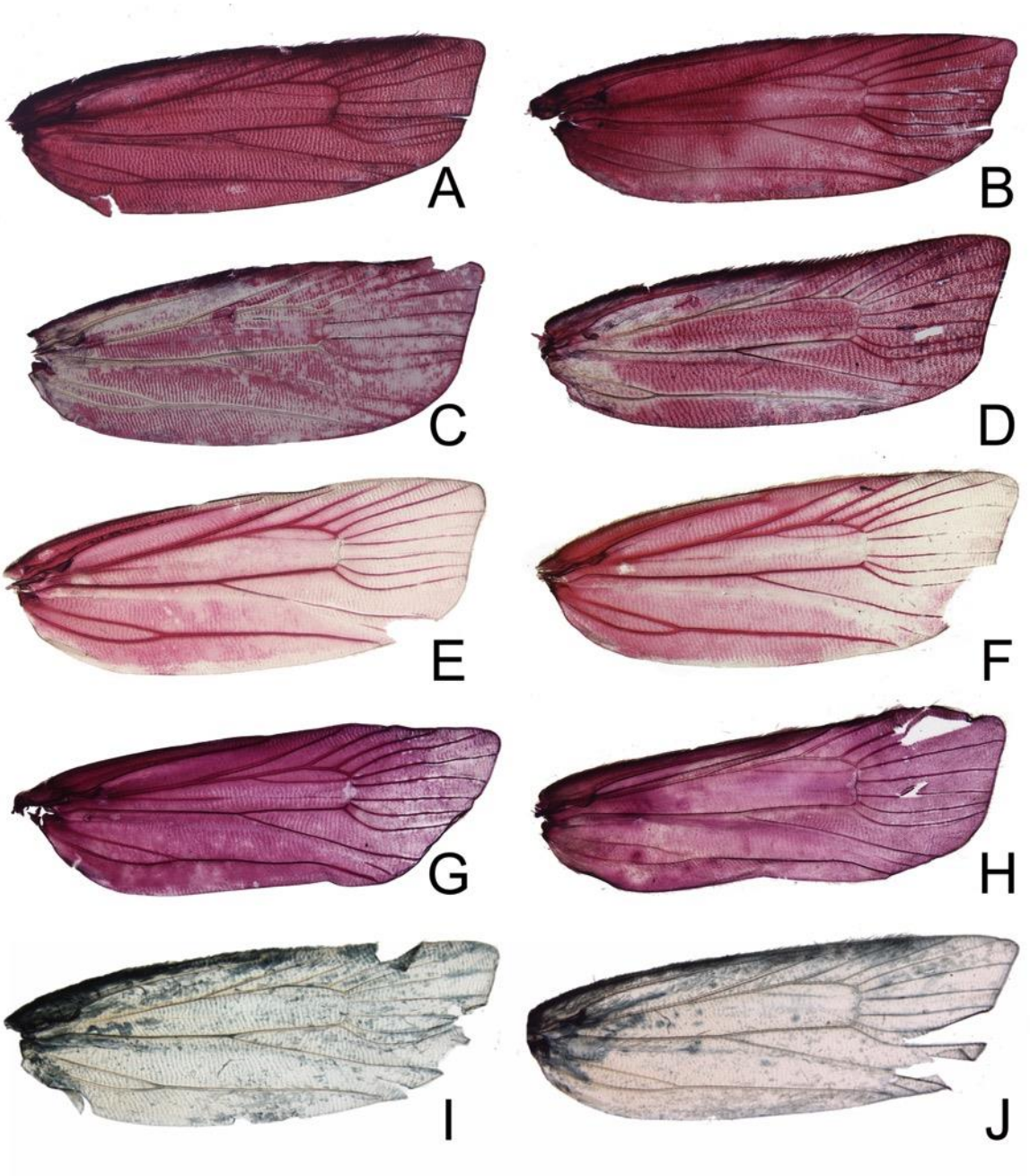

Figure 2.4 Wings stained with Safranin O, acidified eosin Y, Double Stain, and chlorazol black E.

Notes: A-H wings stained for 45 minutes (left) and 90 minutes (right). I-J wings stained for 1 minute. A-B, ethanolic safranin O. C-D, aqueous safranin O. E-F, acidified ethanolic eosin Y. G-H, Double Stain. I, aqueous chlorazol black E. J, ethanolic chlorazol black E.

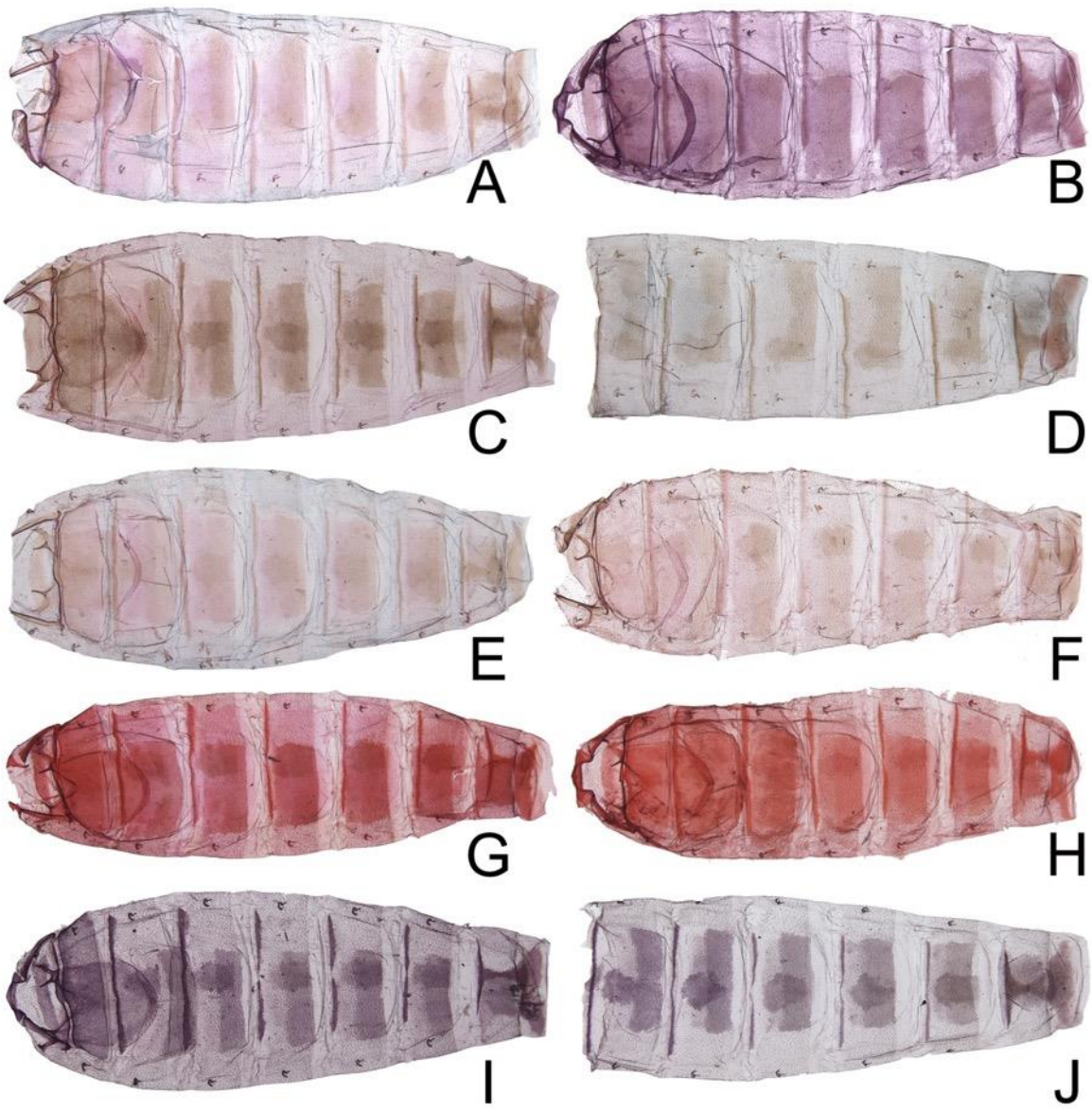

Figure 2.5 Abdomens stained with acid fuchsin, eosin Y, lignin pink, mercurochrome, and safranin O.

Notes: Abdomens stained for 45 minutes in ethanolic (left) and aqueous (right) solutions. A-B, acid fuchsin. C-D, eosin Y. E-F, lignin pink. G-H, mercurochrome. I-J, safranin O.

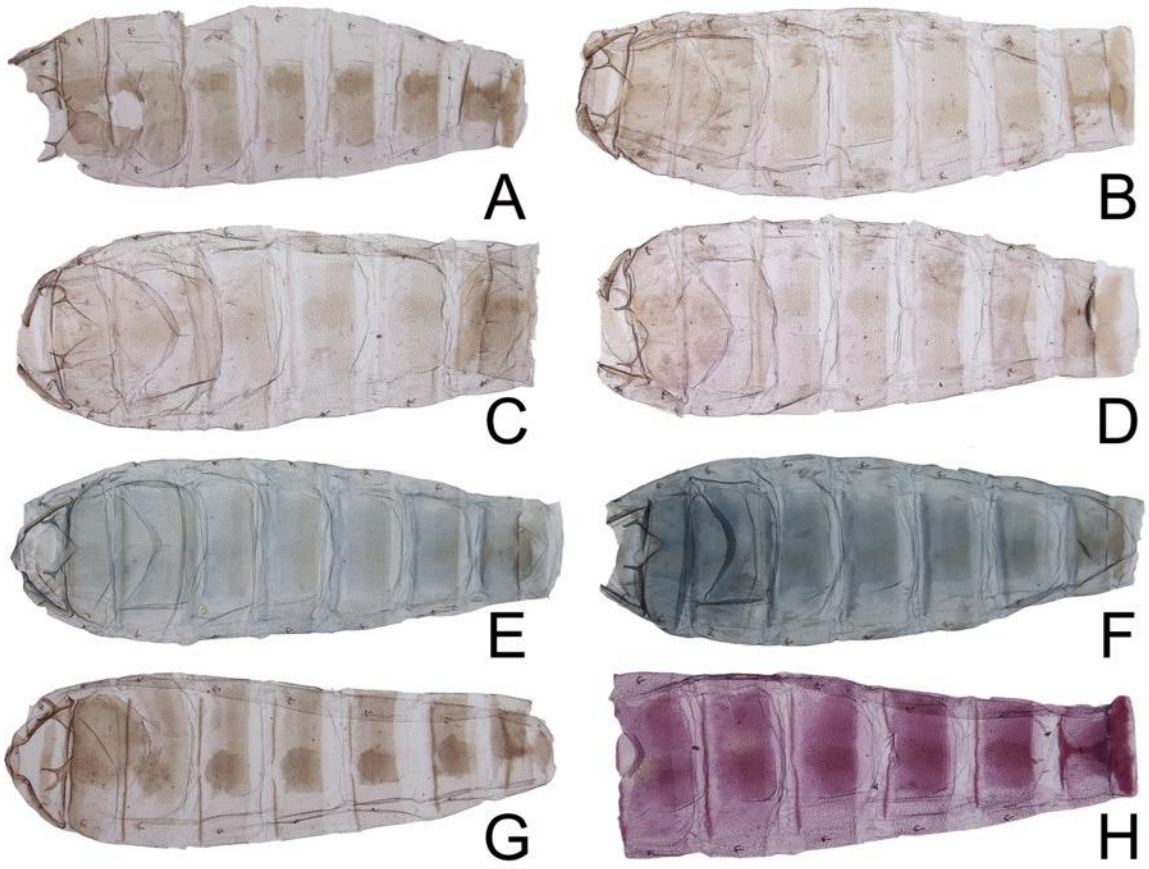

Figure 2.6 Abdomens stained with orange G, rose bengal, chlorazol black E, acidified eosin Y, and double stain.

Notes: A-F abdomens stained in ethanolic (left) and aqueous (right) solutions. A-D and G-H stained for 45 minutes. E-F stained for 1 minutes. A-B, orange G. C-D, rose bengal. E-F, chlorazol black E. G, acidified ethanolic eosin Y. H, Double Stain.

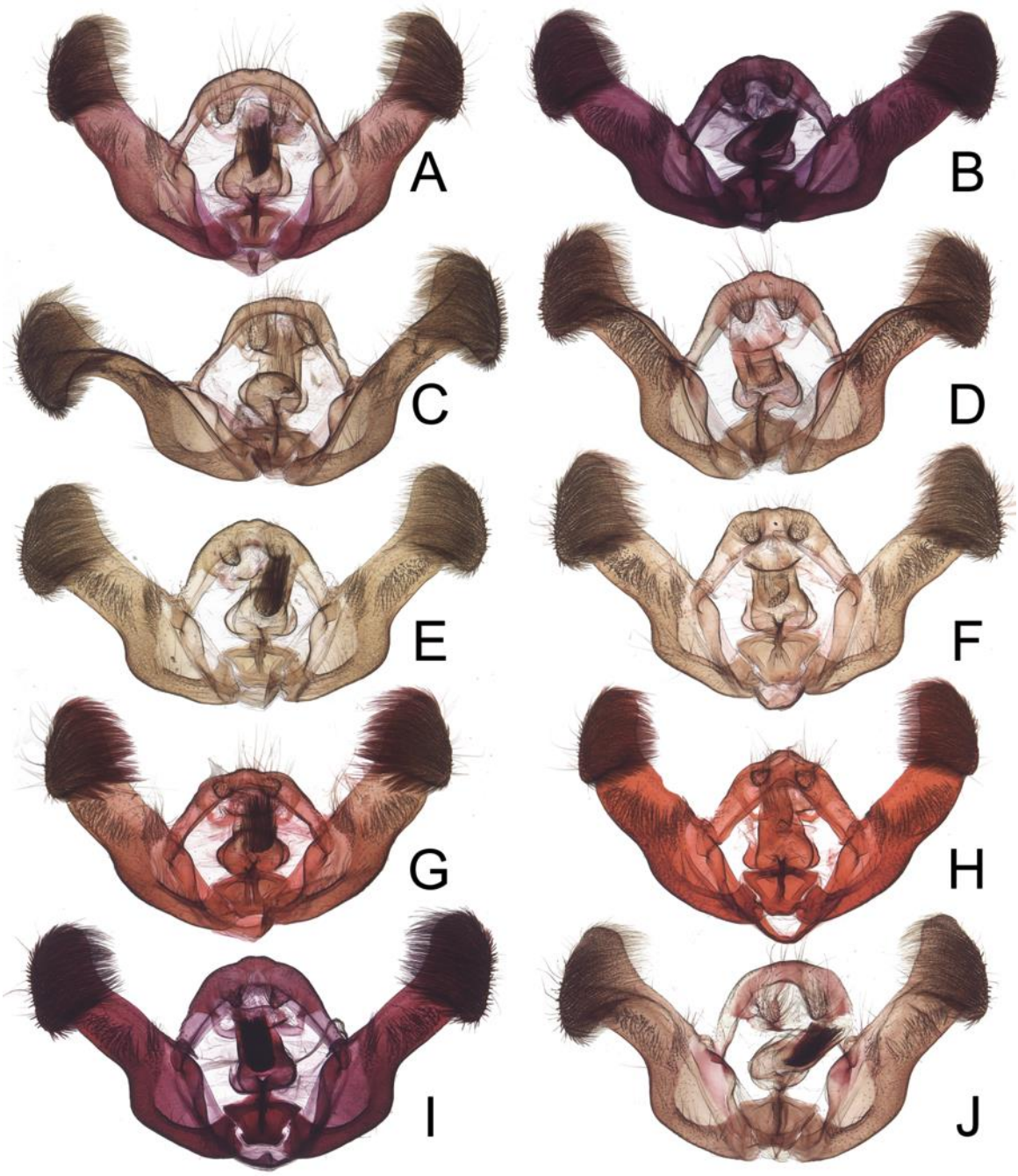

Figure 2.7 Male genitalia stained with acid fuchsin, eosin Y, lignin pink, mercurochrome, and safranin O.

Notes: Genitalia stained for 45 minutes in ethanolic (left) and aqueous (right) solutions. A-B, acid fuchsin. C-D, eosin Y. E-F, lignin pink. G-H, mercurochrome. I-J, safranin O.

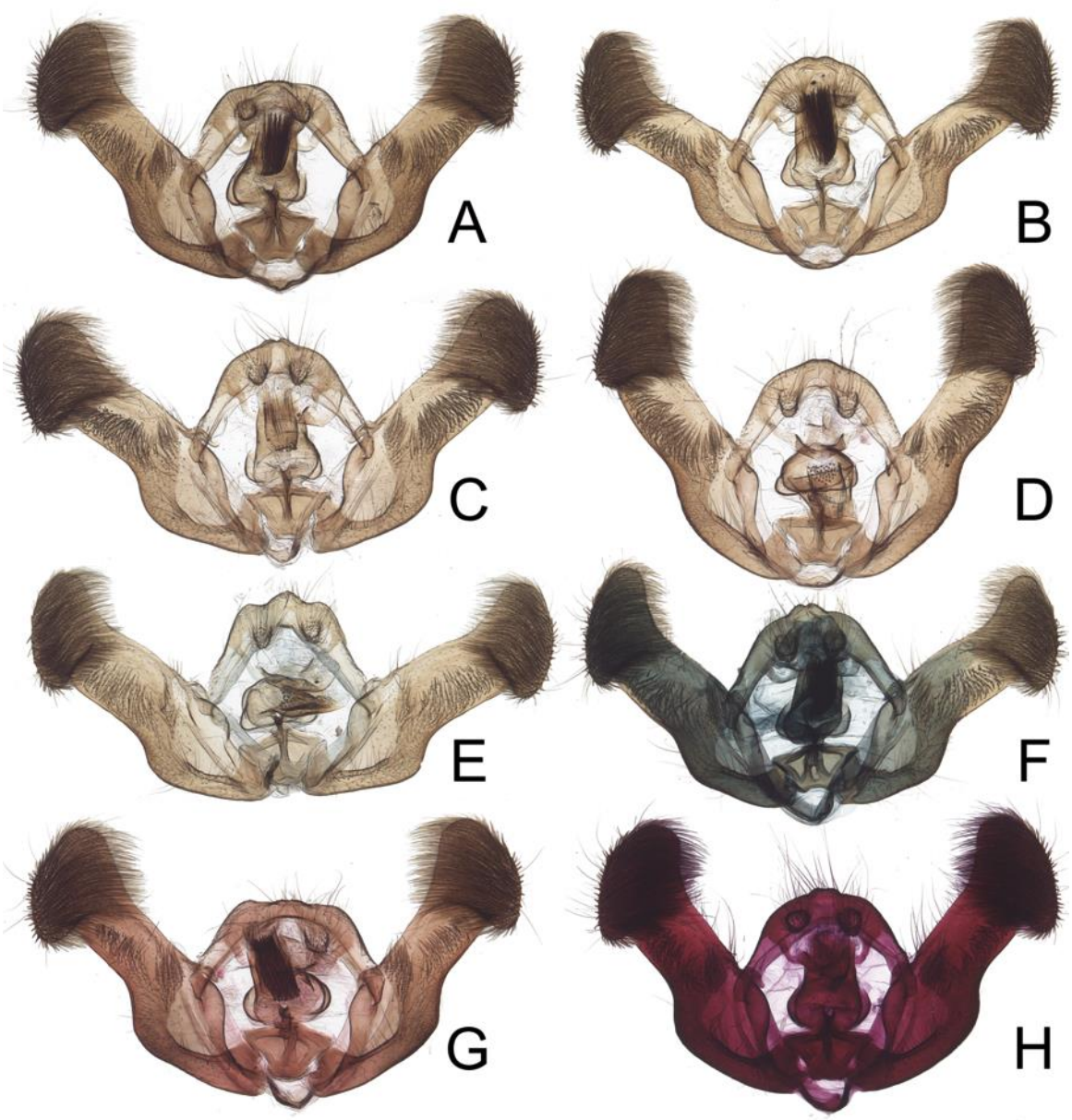

Figure 2.8 Male genitalia stained with orange G, rose bengal, chlorazol black E, acidified eosin Y, and Double Stain.

Notes: A-F genitalia stained in ethanolic (left) and aqueous (right) solutions. A-D, G-H stained for 45 minutes. E-F stained for 1 minutes. A-B, orange G. C-D, rose bengal. E-F, chlorazol black E. G, acidified ethanolic eosin Y. H, Double Stain.

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CHAPTER III

A NON-TRADITIONAL APPROACH TO UNDERSTANDING PHYLOGENETIC RELATIONSHIPS WITHIN TORTRICIDAE (LEPIDOPTERA)

Introduction

Historical classification of Tortricidae

Adults of the family Tortricidae are characterized by several morphological characters: Head with ventral-facing scale tufts beneath the antennae; frons with appressed, short, dorsally-oriented scales; labial palps stout, porrect and sinuate or slightly upcurved, terminal segment short, never long and tapering; proboscis well developed and without scales at the base; maxillary palps reduced, usually not visible without dissection; ocelli and chaetosemata almost always present; papillae anales (ovipositor lobes) always flat, although surfaces face laterally in some Eucosmini (Horak, 2006). Morphology of the papillae anales are the only single apomorphy that unites all Tortricidae; including the Cnephasiini with modified, although still flat, floricomous papillae anales.

Recent molecular studies (Fagua et al., 2016; Regier et al., 2012) have disputed relationships previously predicted by traditional morphology (Horak & Brown, 1991; Kuznetsov & Stekolnikov, 1973, 1984; Razowski, 1976) and by those predicted using biology (Powell, 1964) and pheromones (Safonkin, 2007). Powell (1964) treated the North American tribes of the Tortricinae, and provided biological support for the

monophyly of Tortricinae which included external feeding, or "loss of internal feeding habit[s]", as opposed to the Olethreutinae which tend to bore into stems, seeds, and bark; The latter is a misconception attributed to specious groups like *Eucosma*, *Cydia*, and *Grapholita* that predominate in the Holarctic Region and are primarily monophagous internal feeders of stems, roots, and fruits; most other olethreutine genera in the Olethreutini, which are especially diverse in the southern Hemisphere, and basal Eucosmini are oligophagous and have external feeding habits (Horak & Brown, 1991). Kuznetsov and Stekolnikov (1973) used genital musculature to classify the Tortricidae as having five super tribes (Sparganothidii, Cochylidii, Tortricidii, Olethreutidii, and Eucosmidii) with 12 tribes distributed within. A decade later, Kuznetsov and Stekolnikov (1984) adjusted their phylogenetic estimate, also based on musculature, by expanding the tribe count to 17, adding the supertribes Gatesclarkidii and Archipidii, and sinking 'Sparganothidii' into Cochylidii. Razowski (1976) suggested Olethreutinae and Tortricinae were sister groups, with three tribes each and the now-Chlidanotinae were included within Archipini (Tortricinae) as the subtribe 'Chlidanotina'. Horak and Brown (1991) were the first to recognize three subfamilies, and provided strong evidence for the monophyly of Olethreutinae and the new subfamily 'Chlidanotinae', and likewise indicated that Tortricinae and tribes of Olethreutinae are paraphyletic, comprised of ancestral and derived forms. Additionally, Horak and Brown (1991) provided a detailed review of the biogeography, morphology, and biology the 22 tribes within the Tortricidae, three of which were in Chlidanotinae, 11 in Tortricinae, and eight in Olethreutinae. Lastly, Safonkin (2007) provided the first phylogenetic tree which

recognized the three subfamilies and 23 tribes (the additional tribe being Ramapesiini in Tortricinae), with the Olethreutinae basal to sister groups Tortricinae and Chlidanotinae.

Regardless of the aforementioned advancements made in estimating subfamily level relationships, morphological studies have so far failed to provide compelling evidence regarding the phylogenetic relationships of the tribes within the three subfamilies (Regier et al., 2012). Powell's (1964) treatment used only North American representatives of Tortricinae. Kuznetsov and Stekolnikov (1973, 1984) and Razowski (1976) covered the Palearctic taxa, using a narrow range of characters and, unlike Powell (1964), did not indicate the characters that supported specific clades; additionally, many taxa were defined based on hypothesized shared losses. Many tribes lack defining characteristics that unite all members or demonstrate close relationships with other tribes. e.g. no synapomorphy is known that provides evidence for a close relationship between Tortricini and other Tortricinae tribes (Horak & Brown, 1991). Furthermore, within the Tortricinae, no unambiguous synapomorphy exists that unites specific tribes more closely, or indicates if they be placed elsewhere. The Olethreutinae, however, share two unique features including modified genitalia of the male with fusion of the juxta and caulis (articulated in Chlidanotinae and Tortricinae), and scales on the antennae appearing to be arranged in a single row of similar length scales, as opposed to two apparent rows (the result of a single row which has two lengths of scales), which is the ground plan state for all Ditrysia (Horak, 2006). Presence of cubital pectin is also characteristic of most Olethreutinae, however, these are also present in some 'generalized' Tortricinae (Horak, 2006)

Regier et al. (2012) retains the status of the three subfamilies, with strong support for the monophyly of the sister groups Olethreutinae and Tortricinae; however, the earliest diverging subfamily, Chlidanotinae, is inferred to be paraphyletic with the Polyorthini being basal to all Tortricidae, and Chlidanotini+Hilarographini (node 4, their figure 3) being the sister group to Tortricinae+Olethreutinae (node 5). Fagua et al. (2016) performed a similar molecular-based analysis and also confirmed the monophyly of Tortricinae and Olethreutinae, with a similar tree topology to Regier et al. (2012) and only minor disagreement regarding relationships between three of the tribes. These differences included: (1) the placement of Enarmoniini as a sister group to Olethreutini (0.98 posterior probability), whereas Regier et al. (2012) places the tribe basal to Grapholitini+Eucosmini (<50% bootstrap support in all five analyses), and (2) placement of the Sparganothini+Atterini clade as a sister group to the Cochylini+Euliini+Tortricini+Cnephasiini clade (0.6 posterior probability), whereas Regier et al. (2012) includes them as a sister group to the Archipini+Ceracini clade (two of five analyses with >94% bootstrap support). Based on the strong evidence supporting the monophyly of Tortricinae, Regier et al. (2012) suggest that 'the search for tortricine synapomorphies deserves more effort' because 'convincing morphological synapomorphies have not been found'.

Objectives

Objectives of this study included: (1) to determine agreement and disagreement between a molecular-based phylogenetic estimate (Regier et al., 2012) and one produced using a combination of novel non-traditional characters in addition to traditional

characters; and (2) to identify and provide a descriptive account of non-traditional character distribution across all tribes.

Methodology

Material examined

In this study, approximately 500 specimens were examined from the United States National Museum (USNM) and the Mississippi Entomological Museum (MEM). All 22 tortricid tribes recognized by Brown et al. (2005) were represented. Specimens examined were collected from throughout the world, including Palearctic, Oriental, Australian, Nearctic, and Neotropical regions. Species from eight families, Acrolophidae, Galacticidae, Gelechiidae, Glyphidoceridae, Oecophoridae, Tineidae, Urodidae, and Yponomeutidae, all basal to the Tortricidae, were selected as outgroups.

Preparations of whole body mounts

A total of 154 dissections were prepared and slide mounted, of which 93 were whole body dissections including wings and genitalia; these represented 58 genera and 70 species of tortricids, in addition to the eight outgroup species (Table 3.1). Two species, *Epinotia nisella* (Clerck, 1759) and *E. solicitana* (Walker, 1863) were represented by three specimens each. All dissected specimens were assigned unique dissection numbers that were included on the slides and specimen, or on the pin that included data labels for whole body mounts. Whole body dissections are separated onto two slides, each slide for a single specimen receives the same number and a suffix of 'A' or 'B'. Records or preparations were maintained digitally and also in a hard copy dissection book with records providing species name, date of dissection, specimen sex, collection locality, the

preparer's name, and the unique dissection number.

Methods for dissection of descaled specimens and subsequent slide mounting of whole bodies followed Lee and Brown (2006) with a few modifications. The left pair of wings were removed from the body and dry mounted under a coverslip, which was affixed to the slide by three spots of Elmer's ® white glue. For larger wings, or those with large scale tufts, three vinyl props were affixed to the slide with a small amount of euparal or Elmer's® glue, and the cover slip affixed on top. The right pair of wings were removed from the body and cleaned of scales in 20% ethanol using sizes 000 and 00 synthetic and/or Kolinsky sable brushes, after which they were further cleaned in 70% ethanol. Prior to staining, the wings were placed in bleach for one minute to clear remaining scales on the wing. Wings were stained in a 90% ethanolic solution of 1% w/v (weight/volume) eosin Y acidified with glacial acetic acid (final stain pH=4.7) for one hour to accentuate venation. Wings were then dehydrated in 100% ethanol for several hours or overnight. Wing venation preparations were then mounted in Euparal. The abdomen, mesothoracic legs, and metathoracic legs, were separated from the body and, along with the rest of the body still on the pin, were placed in a 10% potassium hydroxide (KOH) solution at room temperature overnight. The body was removed from the pin and the specimen was cleaned of scales in a 10% KOH solution. Further cleaning was performed in 20%, and then 70%, ethanol. The head was detached from the body, and the labial palps and antennae were removed from the head. The pro-, meso-, and metathorax were separated. The genitalia was removed from the abdomen and prepared using methods from Clarke (1941) and Robinson (1976), with modifications to the staining protocol. All body parts were stained for 30 minutes in a 90% ethanolic solution of 1%

w/v eosin Y acidified with glacial acetic acid (final stain pH=4.7), and counter stained with a 70% ethanolic solution of 1% w/v chlorazol black for 1 minute. After staining, body parts were rinsed in 100% ethanol and further cleaned if needed. During this cleaning, one half of the proboscis (i.e. one galea) was removed from the head to be displayed laterally on the slide. Additionally, the tegulae and the mesonotum were removed from the mesothorax. In females, the abdominal pelt was cut longitudinally along the pleuron. All body parts were transferred to 100% ethanol and positioned under glass chips to be dehydrated overnight. Dehydrated body parts were subsequently mounted in Euparal. The entire specimen, including wings, was mounted on two slides, each with two cover slips. The first slide contained the head, head appendages, prothorax, and mesothorax under the first cover slip and, to the right of that, the metathorax, abdomen, and genitalia were placed under the second cover slip. The second slide contained the left pair of wings dry mounted in the center, under cover slips, and the right pair of de-scaled wings under a second cover slip. Both slides were labeled with the unique dissection number, followed by 'A' for the body slide, and 'B' for the wing slide, the mounting media use (Euparal), the location the specimen was collected, the sex, and the species identification.

Imaging

Certain morphological structures were prepared for examination with SEM. Body parts were placed on an aluminum stub with silver paste and were subsequently dried in a desiccator for at least 24 hours. The stub was then coated with two 15 nm layers of platinum in an EMS 150T ES high resolution sputter coater. Specimens were examined

and imaged using a JEOL JSM-6500F Field Emission Scanning Electron Microscope with a low accelerating voltage of either 5kV or 10kV.

Images of whole-body slide preparations were made for non-traditional characters using a Leica stereoscope with Leica Application Suite 3.7.0© and autoformatted with Montage©, Synoptics Ltd.

Examination of characters in dissected specimens

De-scaled moths were examined and using a Leica MZ125 stereomicroscope and a Leica DM 2000 LED compound microscope. An ocular micrometer in the stereomicroscope was used for measurements. The width between the antennae relative to the width of the anterior most patch of scale sockets between the antennae, is provided as a ratio (Fig. 3.4C). For characters involving scale arrangement (7, 8, 9, 12, 14, 15, 17, 18, and 50), specimens were viewed on a low magnification (Usually 5x, sometimes 2.5x) as the characters can be difficult to interpret at high magnifications.

Three specimens each of *E. nisella* and *E. solicitana* (Olethreutinae: Eucosmini) were available for comparing intra-specific variation. Intra-specific variation, if present, is described in the results. Sexual variation was compared by examining whole mounts of both sexes for the following species: Cochylini [Euliini]: *Orthocomotis sp.,* and *Bonagota salubricola; Aethes rubigana* (Cochylini); *Choristoneura rosaceana* (Archipini), and *E. nisella* (Eucosmini). Therefore, unless stated with the character description results, characters did not exhibit intra-specific or sexual variation.

Terminology in Oseto and Helms (1976) as modified by Kristensen (2003) was used for structures on the descaled head, prothorax, mesothorax, and metathorax. Terminology from Wootton (1979) will be used for describing wing venation

Phylogenetic analysis

In total, 52 morphological characters (Table 3.2) and 135 character states were scored for all 83 taxa (Table 3.3); of them, 38 were binary and 14 were multistate. These characters represented 25 traditional characters, e.g., genitalia, antenna, wing venation and pattern, etc., and 27 non-traditional characters of the endo- and exoskeleton. Characters were coded for various body parts including: Head (nine characters and 30 states), thorax (22 characters and 60 states, of which three characters and 11 states involved the wings, 14 characters and 34 states involved the legs, and the remaining were on the thoracic segments), and abdomen (21 characters and 45 states, of which 18 characters and 39 states involved the genitalia, the remaining three characters and six states were on other parts of the abdomen).

Using the morphological data matrix, the parsimony criterion in PAUP 4.0 (Swafford, 2003) was used to execute a heuristic search with a random 'addition sequence' on 1000 replicates. The tree bisection-reconnection algorithm was used for branch swapping. The ACCTRAN character optimization algorithm was used, however, DELTRAN character optimization has been suggested to be more appropriate for morphological characters (Agnarsson & Miller, 2008); because of this, the parsimony analysis was also run under the DELTRAN condition, which did not produce a different tree topology.

A total of 14 traditional characters were given a weight of '2' as they are tested, reliable synapomorphic characters that define the three subfamilies, and several tribes (Horak & Brown, 1991). Individual weighting of three characters (43, 47, and 49) that are traditional synapomorphies for the Atterini, Tortricini, and Chlidanotini, did not change

the tree topology and thus were not weighted in the final analysis. These characters were only present in members of the tribe for which the character was a synapomorphy; the other weighted tribal characters (42, 44, 45, 46, and 48) were present in other taxa not recognized as members of the associated tribe. For example, character 44 (presence or absence of socii with large scaled lobes) is a synapomorphy for Sparganothini, however it is also present in both the atterines, and two species of Euliini (*Orthocomotis* and *Bonagota salubricola*). In total, 4,264 codings were provided in the data matrix (52 characters for 82 taxa), with 107 of these missing and coded as '—'. Characters were coded as 'missing' if the specimen was missing a body part or if the character was difficult to interpret due to being obscured on the slide, or not visible. In these instances, characters that could not be seen for any given species are described in the results for each character.

Using Mesquite's (Maddison & Maddison, 2015) "edit by hand" function, a tree (Fig. 3.3) based on topology of Regier et al. (2012) was constructed using all 82 taxa (length= 411 steps, CI=0.2019, RI=0.5832). Characters were mapped onto this tree using WinClada (Nixon, 1999) to identify homoplasious apomorphies and synapomorphies for currently recognized groups. Tribes were placed, relative to each other, based on Regier et al. (2012) except in cases where Regier et al. (2012) did not sample them, such as Epitymbiini, Shoenotenini, and Gatesclarkeanini; in these cases, they were placed relative to other tribes based on Fagua et al. (2016). Within the Tortricinae, *Orthocomotis* (Cochylini [Euliini]), *Tortrix viridana* (Tortricini), and *Tinacrucis* (Atterini) were not included in Regier et al. (2012) or Fagua et al. (2016). *Orthocomotis* is one of five other [Euliini] and was therefore placed basal to the other [Euliini], whereas Tortrix and

Tinacrusis were placed in their respective tribes that previously included only one taxon. Several taxa in the Olethreutinae belong to genera that were not sampled in Regier et al. (2012) or Fagua et al. (2016), and these were placed relative to other taxa based on the weighted morphological tree (Fig. 3.1). For example, *Larisa subsolana* (Grapholitini) was placed in the Olethreutini as it was not sampled in the aforementioned studies. Species of *Epinotia* were also arranged relative to each other based on the weighted morphological tree. Three Olethreutinae (*Pelochrista matutina, Eucosma radiatana, Gatesclarkeana idia*) belong to genera that were not sampled in Regier et al. (2012), however were sampled in Fagua et al. (2016) and thus their placement is based on the latter study.

Classification

Classification follows that of Horak (2006), whereby Gatesclarkeanini is synonymized with Olethreutini, and Regier et al. (2012) whereby Euliini is synonymized with Cochylini, and Bactrini and Endotheniini are synonymized with Olethreutini (Gatesclarkeanini was not included in Regier et al. (2012). However, the alternative higher classification of Brown et al. (2005), and used by Fagua et al. (2016), is recognized for the four tribes synonymized by Regier et al (2012) by including these tribes in brackets in text and figures, for example: *Bonagota salubricola* (Cochylini [Euliini]) and *Bactra maiorina* (Olethreutini [Bactrini]).

Results

Analysis of morphological data

The phylogenetic analysis was based on the morphological data matrix (Table 3.3) with 14 weighted characters and yielded five equally parsimonious trees (length 389 steps, CI=0.2571, RI=0.7051) that resulted in a monophyletic clade of all Olethreutinae, a paraphyletic clade of Tortricinae, and a polyphyletic clade of the Chlidanotinae. A strict consensus (Fig. 3.1) of these trees collapsed four nodes (length 392 steps, CI=0.2551, RI= 0.7021), producing two trichotomies (one within the outgroup, and another within Archipini+Epitymbiini). The analysis was run a second time with all characters weighted equally and yielded six equally parsimonious trees (length=345, CI=0.2406, RI=0.6704). A 50% majority rule consensus (Fig. 3.2) of these trees collapsed 16 nodes (length=360, CI=0.2306, RI=0.6516), producing 12 polytomies. The handmade tree topology (Fig. 3.3, length=411, CI=0.2019, RI=0.5874) based on the phylogeny produced by Regier et al. (2012) shows character distribution for synapomorphic and homoplasious characters for all clades, however excludes character information for terminal branches (individual species) except those for singletons that represent the entire tribe (Schoenotenini, Ceracini, Epitymbiini, Polyorthini, Hilarographini, [Bactrini], [Endotheniini], [Gatesclarkeanini]). A more detailed version of this tree, with character state information and character distribution for all clades and terminal branches is included in the Appendix (Figs. 3.9 & 3.10).

Head

The frons is scaled (**character 1)** evenly in all outgroups, Hilarographini, most Chlidanotini, Phricanthini (**node 8)**, Atterini (**node 12)**, Ceracini+Epitymbiini+Archipini

(**node 14)**, Tortricini (**node 19**), and most of the [Euliini] and Cochylini. Scales are lost along a dorsoventral line in the medial area of the frons (Fig 3.4A) in *Auratonota dispersa* (Chlidanotini), Sparganothini (**node 13**), Shoenotenini, Cnephasiini (**node 18**), *B. salubricola* (Cochylini [Euliini]) and *Aethes rubigana* (Cochylini). Most Olethreutinae (**node 7**) also have lost all or many of the scales in the medial area of the frons with two Grapholitini (*Cryptophlebia illepida* and *Grapholita sp.*) and one Eucosmini (*Spilonota ocellana*) being exceptions. This character could not be seen for *Rhopobota naevana* (Eucosmini).

The frons also has a scaleless tract between the antennal sulci (Fig 3.4A; **character 2**) in the majority of tortricids and all outgroups, exceptions being Polyorthini and four Eucosmini (*Epinotia transmissana*, *E. trigonella, Pseudexentera costomaculana,* and *Epiblema scudderiana*). This character could not be seen for *Eucosma radiatana* (Eucosmini).

Some tortricids possesses a ring of microtrichia surrounding the antennal socket (Fig. 3.4B; **character 3**), these include the Phricanthini (**node 8**), *Anacrusis nephrodes* (Atterini), and [Bactrini]; this character is absent all other tortricids and the outgroups.

The anterior-most area with scale sockets on the vertex, between the antennal sulci, varies in width and its relationship to the width between the antennal sockets also varies (Fig. 3.4C, **character 34**). Relative to the width between antennal sockets, the scaled area is largest in *Dichomeris ligulella* (Gelechiidae) with a 1.0 ratio. The next largest, with a ratio of 0.9, are Yponomeutidae, Tineidae, and *C. rosaceana* (Archipini). All other Tortricidae have a ratio less than or equal to 0.8, with the bulk of those less than 0.5 within the Olethreutinae. The smallest ratio found in the Tortricinae is 0.5 and present

in *Phricanthes asperana* (Phricanthini), and three of the five [Euliini] (now Cochylini): *Orthocomotis sp.*, *E. ministrana,* and *P. orphnoxantha.* Aside from Hilarographini, the smallest ratio (0.4) is only found in Olethreutinae, including one Eucosmini (*C. unica*), and four Olethreutini: (*Episimus tyrius, Pseudosciaphila duplex, Olethreutes fasciatana,* and *Gatesclarkeana idia* [Gatesclarkeanini]). This character exhibits intra-specific variation within the three specimens of *E. nisella* whereby *E. nisella* MEM 698 and 697 have ratios of 0.5 and MEM 699 has a ratio of 0.6. This character could not be seen for Oecophoridae, Glyphidoceridae, Sparganothini, *A. mediana* (Epitymbiini), *P. cyclopiana* (Olethreutini), two Grapholitini (*L. subsolana,* and *Grapholita sp.*) and six Eucosmini (*R. buoliana, E. infuscana, E. solicitana* (MEM 733), *Rhopobota naevana*, *Gypsonoma sp.* and *Pseudexentera cressoniana*).

Within the head, a sclerotized plate known as the sitophore forms the 'floor' of the sucking pump (Kristensen, 1998). The apical margin of the sitophore varies and can be angled, straight, curved, or concave (Fig. 3.5C, **character 4**). The outgroups possess all but the angled condition, and the Chlidanotinae possess only straight and curved margins. The Olethreutinae (**node 7**) have angled, straight, and curved margins, but it is apparent that the ancestral condition is 'straight.' However, many taxa have an angled margin, these include: Olethreutini: [Bactrini]*, Olethreutes fasciatana*; Eucosmini: *P. costomaculana, Epiblema scudderiana, Epinotia nisella, E. johnsonana,* and *E. trigonella.* Several Olethreutinae also have curved margins, including *Cryptaspasma bipenicilla* (Microcorsini), *Phaecasiophora niveiguttana* (Olethreutini)*, Lobesia aeolopa,* Enarmoniini, *Spilonota ocellana* (Eucosmini), *P. cressoniana*, *Epinotia medioplagata,* and *Catastega aceriella.* This character could not be seen for *Anisogona mediana*

(Epitymbiini), *Cryptaspasma haplophyes* (Microcorsini), *Hedya dimidiana* (Olethreutini), *Grapholita sp.* (Grapholitini), and *Epinotia infuscana* (Eucosmini). The Tortricinae (**node 6**) have all four conditions, however, only two taxa have a concave apical margin; these include *Cerace xanthocosma* (Ceracini), and *A. rubigana* (Cochylini). Within the Tortricidae, five members have curved margins, these include: *Palaeotoma styphelana* (Shoenotenini), Cnephasiini (both species), *Acleris variana* (Tortricini), and *Eulia ministrana* (Cochylini [Euliini]). Both species of *Choristoneura* (Archipini) have angled margins. All other Tortricinae have straight margins.

The sitophore, as defined by Kristensen (1998), has two groups of chemoreceptors that appear as rounded sensilla (Fig 3.5B, **character 5**) that occur at the posterior and anterior ends of the sitophore. The posterior area can either have a single pair, or two pairs of sensilla, whereas the anterior area can have no sensilla, a single pair, or pairs of three, four, 5-7, or ten. Within the outgroups, Yponomeutidae possess two pairs posterior and two groups of 5-7 anterior, which is the most common state for Tortricidae. Oecophoridae, Glyphidoceridae, Gelechiidae, and Urodidae all have a two single sensilla both posterior and anterior, whereas Galacticidae has two pairs posterior and two groups of three anterior. All Chlidanotinae (Polyorthini + **node 3**) share the same condition as Yponomeutidae (2 pairs posterior, 2 groups of 5-7 anterior). Within the Tortricinae, all members have the aforementioned condition, with the exception of the following: *A. nephrodes (*Atterini) with two groups posterior, two groups of ten anterior; *Palaeotoma styphelana* (Shoenotenini) and *A. mediana* (Epitymbiini) with two pairs posterior, two groups of three anterior; *Argyrotaenia alisellana* (Archipini) (two pairs posterior, 2 pairs anterior); *A. rubigana* (Cochylini) and *E. mexicana* (Cochylini [Euliini]) with two pairs

posterior, none anterior. Like the tortricines, Olethreutinae is largely dominated by taxa with two pairs posterior and two groups of 5-6 anterior, exceptions to this are: *Paralobesia cyclopiana* (Olethreutini), *Hedya separatana* (Olethreutini), *Cryptophlebia illepida* (Grapholitini), *Crocidosema unica* (Eucosmini), *Rhopobota naevana* (Eucosmini), *Catastega aceriella* (Eucosmini) and *Epinotia trigonella* (Eucosmini) with two pairs posterior and two groups of ten each anterior; [Bactrini]+[Endotheniini] (**node 26**) with two pairs posterior and two pairs anterior; *Larisa subsolana* (Grapholitini), *Rhyacionia buoliana* (Eucosmini) with two posterior and two groups of three anterior; *Pelochrista matutina* (Eucosmini) with one elongate pair posterior and 4 irregular sensilla anterior. This character could not be seen for Acrolophidae, Tineidae, *Ancylis nubeculana, Grapholita sp.,* and *Epiblema scudderiana*.

Ocelli are absent in all outgroups sampled (**Character 22**), but they are rarely absent from tortricids (**node 1**) (Horak & Brown, 1991). The only two tortricid taxa sampled that do not possess ocelli are *Palaeotoma styphelana* (Shoenotenini) and *Pseudomeritastis orphnoxantha* (Cochylini [Euliini]). Some species have very small, reduced ocelli that were only visible due to enhanced staining, such as *Netechma egens* (Cochylini [Euliini]. The remaining [Euliini] (now Cochylini) have small but noticeable ocelli, except the aforementioned *P. orphnoxantha*. This character could not be seen for *Amorbia humerosana* (Sparganothini).

All Tortricidae, with one exception, have the lower scales on the frons directed dorsally rather than ventrally as seen in all outgroups (**Character 40**). *Aethes rubigana* is the sole exception with medial scales on the frons directed ventrally, however, laterally they are directed dorsally.

All Chlidanotini (**node 4**) have antennae with distal flagellomeres compressed (appressed scales) (**Character 49**), which is absent in all other taxa.

Prothorax

The anterodorsal margin of the propleuron (Fig. 3.5A, **character 6**) has a slender apophysis in all Olethreutinae, excluding two Eucosmini, *R. buoliana* and *Gypsonoma sp*. The apophysis is absent in many Tortricinae, including all Chlidanotini (**node 4**), Atterini (**node 12**), Shoenotenini, Ceracini+Epiytymbiini+Archipini (**node 14**), Cnephasiini (**node 18**), three [Euliini] (now Cochylini; *E. ministrana, B. salubricola,* and *Netechma egens*), and the remaining Cochylini (**node 21**). The apophysis in *P. epidesma* (Polyorthini) is so small that it may be considered absent or extremely reduced, whereas the one in *T. jonesi* (Hilarographini) is a distinctive slender apophysis with a club at the end, more similar to those found in the Olethreutinae. Although this character was coded as 'present' or 'absent', there is a clear difference in the apophyses found in the tortricines and those in most of the Olethreutines. Those in the tortricines (and *Gatesclarkeana idia*) are broader and square-like (Fig. 3.6 A) and sometimes extending posteriorly, whereas those in Olethreutinae are long and slender (Fig. 3.5A), tending to extend medially. Tortricine apophyses can be misinterpreted as not being present because of the tendency to face posteriorly (towards the person viewing the structure) making them appear absent (Fig. 3.5 B *P. flexilineana*). Only two outgroups, Galacticidae, and Gelechiidae, have apophyses, and both are reduced and point posteriorly.

Mesothorax

Attached to the mesonotum are a pair of tegulae, the dorsal surface of which can have scales arranged in rows (Fig. 3.6C **character 12**), or randomly. Rows are present in 55 of the 82 taxa sampled, and found in all three subfamilies, and outgroups. Acrolophidae is the only outgroup taxon without the 'row' arrangement. All Chlidanotinae except *Heppnerographa tricesimana* (Chlidanotini) have a 'row' arrangement; *H. tricesimana* appears to have subtle evidence of rows but was coded as 'random'. Within the Tortricinae, those with 'random' scale arrangement include Sparganothini+Atterini+Shoenotenini+Ceracini+Epitymbiini+Archipini (**node 10**), *Cnephasia alticolana* (Cnephasiini), and *Eugnosta busckana* (Cochylini). All remaining Tortricinae (Phricanthini, Tortricini, all [Euliini], and two Cochylini: *E. mexicana, A. rubigana*) have scale sockets arranged in rows. Within the Olethreutinae, those with 'random' scale arrangement include *Episimus tyrius* (Olethreutini), Enarmoniini (**node 28**), and two species of Eucosmini, *R. buoliana* and *E. solicitana*. Like *H. tricesimana, Ancylis myrtillana* appears to have weak evidence of broad rows that have converged, however they are not definite and were therefor coded as 'random'. All other Olethreutinae have scale sockets arranged in rows, which include all members of Microcorsini and Grapholitini, in addition to members of Olethreutini and Eucosmini not mentioned above. This character could not be seen for *Pelochrista matutina, Eucopina tocullionana, Crocidosema unica,* and *S. ocellana*.

The mesothoracic furca (Fig. 3.6B) possesses two spiniform processes medially, on each side, that project anterodorsally. This process can have a dentate projection pointing posterolaterally either on the spiniform process, at the base of the process, or it

can be absent (**character 13**). Within the outgroups, this projection is at the base of the process in Acrolophidae, Yponomeutidae, Glyphidoceridae, and Galacticidae, and is absent in Tineidae, Oecophoridae, and Urodidae. Within the Chlidanotinae, *P.* epidesma (Polyorthini) and *H. tricesimana* (Chlidanotini) have the projection present at the base of the process, and the remaining three chlidanotines lack a dentate projection. All tortricines have a dentate projection, and most have it at the base of the spiniform process, although some have the projection on the process, including: Phricanthini (**node 8**), *N. egens* (Cochylini [Euliini]), and two Cochylini (*A. rubigana,* and *E. busckana*). The dentate projection is absent in two Olethreutinae (Olethreutini: *L. aeolopa*, and Grapholitini: *Grapholita sp.*), and present at the base in nearly all other members; six Olethreutinae have the projection on the spiniform process (Olethreutini: *H. dimidana*, *O. fasciatana*, [Bactrini]; Grapholitini: *L. subsolana*, and *C. pomonella*.)

Metathorax

Posterior to the metascutellum, is the abdominal euphragma, which has lateral flanges (Fig. 3.6D, **character 16**) in nearly all Tortricidae (**node 2**), the exceptions being: *Trymalitis climacias* (Chlidanotini) and *C. rosaceana* (Archipini). All outgroups, excluding Yponomeutidae, lack lateral flanges. This character could also not be seen for Galacticidae, *Tinacrucis sp.* (Atterini), *C. xanthocosma* (Ceracini), *N. egens* (Cochylini [Euliini]), *P. cyclopiana* (Olethreutini), and three Eucosmini (*C. unica, E. solicitana* 721, and *Gypsonoma sp.*).

Many Tortricidae have microtrichia on the metascutellum (Fig. 3.7, **character 23**), a trait that is absent in all the outgroups. With the exception of *H. tricesimana* (Chlidanotini), all Chlidanotinae lack microtrichia on the metascutellum. Presence and

absence of microtrichia is almost evenly distributed throughout Tortricinae, with the following eleven taxa having microtrichia present: Phricanthini (**node 8**), *A. nephrodes* (Atterini), *A. humerosana* (Sparganothini), *P. styphelana* (Shoenotenini), *C. rosaceana* (Archipini), *Decodes basiplagana* (Cnephasiini), *Tortrix viridana* (Tortricini), *Orthocomotis sp.* (Cochylini [Euliini]), *B. salubricola* (Cochylini [Euliini]), *N. egens* (Cochylini [Euliini]), and *A. rubigana* (Cochylini). Those in *N. egens* are sparse, however broadly distributed from the mediolateral margins to the posterior margin. In *A. nephrodes the* microtrichia are restricted to small areas on the anterolateral margins. In *A. rubigana* the microtrichia have a unique posterolateral distribution, extending from the posterior corners narrowly along the posterior margin. Microtrichia are distributed throughout all Olethreutine tribes except Microcorsini. The following Olethreutinae have microtrichia: Olethreutini: *Pseudosciaphila duplex, Paralobesia cyclopiana, Phaecasiophora niveiguttana, L. aeolopa,* [Gatesclarkeanini], and [Bactrini]; Enarmoniini (**node 28**); Grapholitini: *Cryptophlebia illepida*, *Cydia pomonella*, *Grapholita sp.*; Eucosmini: *Pelochrista matutina, Eucosma radiatana, Epinotia infuscana, E. solicitana*, *E. transmissana, E. nisella, E. johnsonana*, *Gretchena deludana, Crocidosema unica, Spilonota ocellana, Pseudexentera cressoniana, P. costomaculana,* and *Epiblema scudderiana*. This character appeared to exhibit variation between the sexes, with females tending to have more microtrichia that are slightly larger than those of the males. The female *A. rubigana* (Cochylini) has small microtrichia sparsely distributed in the posterior corners of the metascutellum, whereas the male only has a few (<10) small microtrichia isolated to the posterior corners not extending medially or towards the lateral margins as in the female. Microtrichia are present in both sexes of

Orthocomotis sp. and *B. salubricola* (Cochylini [Euliini]) however the males have fewer, sparsely distributed microtrichia, which cover a smaller area than the female. Microtrichia appear to be equally distributed in both sexes of *C. rosaceana* (Archipini). The female *E. nisella* (Eucosmini) has very small microtrichia that are sparsely, although evenly, distributed in the anterior corners of the metascutellum; males (MEM 699 and 697) have even fewer microtrichia that are more reduced in size relative to the already small microtrichia in the female.

Microtrichia on the metascutellum are occasionally modified to be broader (Fig. 3.5A, **character 35**), similar to those of the metascutum that are involved in locking the anal area of forewing to thorax (Common, 1969).These specialized microtrichia are found only in four taxa: *H. tricesimana* (Chlidanotini), *P. styphelana* (Shoenotenini), *D. basiplagana* (Cnephasiini), and *B. salubricola* (Cochylini [Euliini]).

In addition to microtrichia, the metascutellum has scales, as evidenced by presence of scale sockets (Fig. 3.7B, **character 24**), which are lost in several Tortricinae, one Chlidanotinae (*H. tricesimana*), and Oecophoridae. The following Tortricinae do not have scales on the metascutellum: *Tinacrucis sp.* (Atterini), Shoenotenini, Cnephasiini (**node 18**), and *P. orphnoxantha* (Cochylini [Euliini]).

Legs

Scale socket arrangement on the was coded as either ‘random’ or ‘rows’ (Figs. 3.5B, and 3.6A, **character 7**), both of which are distributed throughout all three subfamilies, with scale sockets being arranged in rows for 51 of the 82 taxa. Acrolophidae was the only outgroup without scale sockets arranged in rows, although the state could not be determined for Oecophoridae. All Chlidanotinae, excluding *T.*

climacias (Chlidanotini) have scale sockets arranged in rows. *T. climacias* appears to have scale sockets weakly organized and maybe not completely random, however there are no distinctive rows, therefor it was coded as being 'random'. Within the Tortricinae, Archipini+Epitymbiini+Ceracini+Shoenotenini+Atterini+Sparganothini (**node 10**), *Orthocomotis sp.* (Cochylini [Euliini]), *P. orphnoxantha* (Cochylini [Euliini]), and *E. busckana* (Cochylini) all have scale sockets arranged randomly. The remaining Cochylini, Tortricini, *D. basiplagana* (Cnephasiini), and Phricanthini have scale sockets arranged in rows. *D. basiplagana* and *T. viridana* 'rows' are not as defined as the other tortricines, such as Phricanthini, however they are weakly arranged into rows, which become more evident toward the distal end of the procoxa. *B. salubricola* (Cochylini [Euliini]) also has weakly developed rows, however, they are more definite than the two aforementioned Tortricinae. Excluding Phricanthini, none of the Tortricinae with 'rows' have those that are well defined and as apparent as those seen in many Olethreutinae. Although sockets are arranged in rows for most (29/40 species) Olethreutinae, both states (random and rows) are represented in all tribes excluding Enarmoniini (sockets arranged in rows). Although Microcorsini (Olethreutinae) have random scale socket arrangement, they are unique in having scale sockets densely arranged with minimal space between sockets (Fig. 3.8B). Taxa that do not have sockets arranged in rows, and were coded as 'random' include: Olethreutini: *Pseudosciaphila duplex,* and [Bactrini]; *Grapholita sp.* (Grapholitini); Eucosmini: *Eucopina tocullionana, Eucosma radiatana, Epinotia solicitana* (all specimens), *Epinotia trigonella, S. ocellana,* and *Catastega aceriella.* All other Olethreutinae have sockets arranged in rows.

Like the procoxa, scale sockets on the profemur can be arranged in rows or randomly (**character 8**), with the former condition being more common (rows in 51/78 species) and distributed throughout all three subfamilies and the outgroup. Within the outgroup, Tineidae, Yponomeutidae, Glyphidoceridae, Gelechiidae, and Galacticidae all have sockets arranged in rows. In the Chlidanotinae, Polyorthini, Hilarographini, and *A. dispersa* (Chlidanotini) all possess sockets arranged in rows whereas the remaining two Chlidanotini (*H. tricesimana,* and *T. climacias*) have sockets arranged randomly. Like scale socket arrangement in the procoxa, the Tortricinae clade including Archipini+Epitymbiini+Ceracini+Shoenotenini+Atterini+Sparganothini (**node 10**) is united by all members having a random socket arrangement. This condition is shared with Cnephasiini, *E. ministrana* (Cochylini [Euliini]), and *E. busckana* (Cochylini). The remaining tortricines have sockets arranged in rows: Phricanthini; Tortricini; *Orthocomotis sp.* and *B. salubricola* (Cochylini [Euliini]); Cochylini: *A. rubigana* and *E. mexicana*. Nearly all olethreutines (35/40 species) have sockets arranged in rows, those that are arranged randomly include five species of Eucosmini: *Rhyacionia buoliana, Eucosma radiatana, Epinotia solicitana, S. ocellana,* and *E. trigonella.* This character could not be seen for *P. orphnoxantha* (Cochylini [Euliini]) and *N. egens* (Cochylini [Euliini]).

Scale sockets of the protibia can be arranged randomly, in rows, elongate groups (Fig. 3.8C), or round groups (Fig. 3.8A, **character 9**). Scale sockets are arranged randomly in Acrolophidae, Tineidae, Yponomeutidae, and Urodidae; sockets are arranged as elongate groups in Oecophoridae, and Glyphidoceridae, and are arranged in rows in Galacticidae and Gelechiidae. Within the Chlidanotinae, sockets are arranged in

elongate groups for Polyorthini, Hilarographini, and *H. tricesimana* (Chlidanotini). The two remaining Chlidanotini have sockets arrange randomly (*T. climacias*) or in round groups in (*A. dispersa*). With the exception of *C. xanthocosma* (Ceracini), all Tortricinae have sockets arranged in elongate groups. Scale sockets in Olethreutinae are arranged in elongate groups in all taxa except some Olethreutini that have sockets arranged in rows (*Phaecasiophora niveiguttana*) and round groups (*Paralobesia cyclopiana*, *H. separatana, H. dimidana, L. aeolopa, G. idia* [Gatesclarkeanini], and *E. hebesana* [Endotheniini]). This character could not be seen for *P. orphnoxantha* (Cochylini [Euliini]) and *Epinotia infuscana* (Eucosmini).

Scale sockets on the first protarsomere can be arranged randomly, as rows, or in elongate groups (**character 10**). Nearly all taxa have sockets arranged in elongate groups with a few exceptions: Galacticidae has sockets arranged in rows, and Tineidae and *C. xanthocosma* (Ceracini) have sockets arranged randomly. This character could not be seen for *P. orphnoxantha* (Cochylini [Euliini]) and *Epinotia infuscana* (Eucosmini).

The protarsomeres can have spiniform setae present on each segment (Fig. 3.8A, **character 11**) or they can be reduced or absent. When present, there are either two, three, or five or more setae on each segment. Each of the four conditions is present in the outgroups, with Acrolophidae and Galacticidae having one spiniform setae per segment, Glyphidoceridae having two per segment, Yponomeutidae, Oecophoridae, and Gelechiidae have five or more per segment and finally, Tineidae lacking spiniform setae on the protarsomeres. Within the Chlidanotinae, Polyorthini, Hilarographini, and two of the three Chlidanotini (*A. dispersa* and *T. climacias*) have reduced setae that are barely visible with a compound microscope (x10 magnification), as such they were coded as

'absent or reduced'. The remaining Chlidanotini, *H. tricesimana,* has three spiniform setae per segment. Most Tortricinae have three spiniform setae per segment, however they are absent in Phricanthini (**node 8**) and Tortricini (**node 19**), and several individuals have five, such as *C. xanthocosma* (Ceracini), *C. pinus* (Archipini), *C. alticolana* (Cnephasiini), *Orthocomotis sp.* (Cochylini [Euliini]), and *B. salubricola* (Cochylini [Euliini]). Nearly all Olethreutinae have three spiniform setae per segment, exceptions being Microcorsini (**node 23**), and three Olethreutini with spiniform setae absent: [Gatesclarkeanini]+[Bactrini]+[Endotheniini] (**node 25**).

The mesofemur can have scale sockets arranged randomly or in rows (**character 14**) with the former condition being more common (rows in 51/78 species) and distributed throughout all three subfamilies and the outgroups. Within the outgroups, Tineidae, Yponomeutidae, Glyphidoceridae, Gelechiidae, and Galacticidae all have sockets arranged in rows. In the Chlidanotinae, Polyorthini, Hilarographini, and *A. dispersa* (Chlidanotini) all possess sockets arranged in rows whereas the remaining two Chlidanotini (*H. tricesimana* and *T. climacias*) have sockets arranged randomly. The Tortricinae clade represented by Archipini+Epitymbiini+Ceracini+Shoenotenini+Atterini+Sparganothini (**node 10**) is united by all members having a random scale socket arrangement. This condition is shared with Cnephasiini, *E. ministrana* (Cochylini [Euliini]), and *E. busckana* (Cochylini). The remaining tortricines have sockets arranged in rows: Phricanthini, Tortricini, *Orthocomotis sp.* and *B. salubricola* (Cochylini [Euliini]), and two Cochylini (*A. rubigana* and *E. mexicana*). Nearly all Olethreutinae (35/40 species) have scale sockets arranged in rows, except five Eucosmini whose sockets are arranged randomly

(*Rhyacionia buoliana,* all three *E. solicitana,* and *S. ocellana*). This character could not be seen for *P. orphnoxantha* (Cochylini [Euliini]), *N. egens* (Cochylini [Euliini]), and two Eucosmini (*Eucosma radiatana* and *Epinotia trigonella*).

Scale sockets on the mesotibia are arranged randomly, in rows, elongate groups, or round groups (**character 15**). Tineidae, Gelechiidae, and Galacticidae have sockets arranged in rows, whereas Urodidae, Oecophoridae, and Acrolophidae have sockets arranged randomly. The two remaining outgroups, Yponomeutidae, and Glyphidoceridae have sockets arranged in elongate groups. Chlidanotinae have sockets arranged in round groups in Polyorthini, Hilarographini, and *T. climacias* (Chlidanotini) or elongate groups in the remaining Chlidanotini (*H. tricesimana* and *A. dispersa*). The majority of Tortricinae (18/25 species) have elongate groups, however five species representing Phricanthini, Atterini, Archipini, and Tortricini (*P. flexilineana*, *Tinacrucis sp.*, *C. rosaceana, A. variana* and *T. viridana*) have round groups, a random arrangement is present in Ceracini, and rows are present in Atterini. Olethreutines exhibit only elongate and round group scale arrangements and, unlike the Tortricinae, the majority of Olethreutinae (29/40 species) have round groups, which includes Microcorsini, all but one Olethreutini (*B. maiorina* [Bactrini]), all Grapholitini, and 15 Eucosmini. Those remaining, which includes Enarmoniini and seven Eucosmini (*Pelochrista matutina, Rhyacionia buoliana, Gretchena deludana, Crocidosema unica, Epinotia transmissana, E. solicitana* (MEM 733), and *S. ocellana*) have elongate groups. This character appears to have some intra-specific variation within *E. solicitana* in which one specimen (MEM 733) has elongate groups, and the other two (MEM 721 and 722) have round groups.

There was no variation with *E. nisella*, however. This character could not be seen in two Eucosmini, *Epinotia trigonella* and *Eucosma radiatana*.

Scale sockets on the metafemur are arranged randomly or in rows (**character 17**). Unlike scale arrangement on the mesofemur (rows in 51/78 species), the 'row' scale arrangement is much less common, being found only in 18 species. The only outgroup with scale sockets arranged in rows is Gelechiidae. All of the Chlidanotinae, and nearly all of the Tortricinae (**node 9**) have scale sockets arranged randomly. The only Tortricinae with sockets arranged in rows are Phricanthini. Within the Olethreutinae, 14 species have scale sockets arranged in rows which includes Microcorsini (**node 23**), three Olethreutini (*E. tyrius, H. separatana,* and *E. hebesana* [Endotheniini]) and nine Eucosmini (*Epinotia infuscana, E. nisella, E. johnsonana, E. medioplagata, Crocidosema unica, Gypsonoma sp., Rhopobota naevana, Pseudexentera cressoniana,* and *P. costomaculana*). Scale sockets on the metafemur are often less densely populated than the meso- and profemur, and therefor can appear randomly arranged; for example, there were fewer scale sockets in Microcorsini and they were arranged in single file rows with lots of space between each scale socket, relative to other Olethreutinae such as *E. nisella* in which scale sockets are closer together and each row is two or three scale sockets wide.

Scale sockets on the metatibia are arranged randomly, in rows, or in round groups (**character 18**). None of the Tortricidae have sockets arranged in rows, however, this is unique to Gelechiidae. Galacticidae and Tineidae have scale sockets arranged in round groups, and the remaining five outgroups have scale sockets arranged randomly. *C. xanthocosma* (Tortricinae: Ceracini) and *Cryptophlebia illepida* (Olethreutinae: Grapholitini) have random scale socket arrangement. All other Tortricidae have scale

sockets arranged in round groups, however, this character could not be seen in *A. dispersa* (Chlidanotinae: Chlidanotini).

Scale sockets on the first metatarsomere are arranged randomly, or in elongate groups (**character 19**). All Chlidanotinae, Olethreutinae, and all but one Tortricinae have sockets arranged in round groups. Ceracini (Tortricinae), Urodidae, and Yponomeutidae have scale sockets of arranged randomly. This character could not be seen in *E. infuscana* (Eucosmini) and *A. dispersa* (Chlidanotinae: Chlidanotini).

The metatarsomeres can have spiniform setae present on each segment (**character 20**) or they can be reduced or absent. When present, there are three, four, or five or more setae on each segment. Tineidae does not have spiniform setae, whereas Acrolophidae and Glyphidoceridae have three, and Yponomeutidae, Oecophoridae, and Gelechiidae have 5 or more. *Polylopha. epidesma* (Polyorthini) and *T. climacias* (Chlidanotini) do not have spiniform setae, however Hilarographini has three, and *H. tricesimana* (Chlidanotini) has 5 or more. All four conditions are present in the Tortricinae, with the most basal group, Phricanthini, and Tortricini having no spiniform setae. Atterini, Ceracini, *Choristoneura* (Archipini), *C. alticolana* (Cnephasiini), and three Cochylini [Euliini] (*Orthocomotis sp., B. salubricola,* and *P. orphnoxantha*) all have five or more setae. Sparganothini, Shoenotenini, Epitymbiini, *A. alisellana* (Archipini), and four Cochylini (*N. egens* [Euliini], *A. rubigana, E. busckana,* and *E. mexicana*) have three spiniform setae. All Olethreutinae have three spiniform setae, with the following exceptions in Olethreutini, [Gatesclarkeanini]+[Bactrini]+[Endotheniini] (**node 25**), which have absent or reduced spiniform setae.

The proleg claw in many Tortricinae (18/25 species) has microtrichia (Fig. 3.8C, **character 21**), that are concentrated near the base. All Olethreutinae have 'simple' claws that are not microtrichiate (Fig. 3.8D). Oecophoridae has microtrichiate claws; whereas all other outgroups have 'simple' claws. The following Tortricinae have microtrichiate proleg claws: Atterini, Shoenotenini, Archipini, Cnephasiini, Cochylini (including [Euliini] except *P. orphnoxantha* which could not be coded), *A. humerosana* (Sparganothini), *T. viridana* (Tortricini). *H. tricesimana* (Chlidanotini) is the only Chlidanotinae with microtrichiate claws. This character could not be seen in *P. orphnoxantha* (Cochylini [Euliini]) and *P. flexilineana* (Phricanthini).

Wings

Some Tortricinae have tufts of scales on the forewing (**character 25**); these include: *D. basiplagana* (Cnephasiini), Shoenotenini, and *P. asperana* (Phricanthini). Polyorthini (Chlidanotinae) and Acrolophidae also have scale tufts on the forewing. All other taxa sampled do not have scale tufts on the wings.

Many Olethreutinae have an ocellus on the forewing (**character 26**), a feature not present in any Tortricinae, Chlidanotinae, or outgroup taxa (except not seen in Glyphidoceridae). Within the Olethreutinae, only one Olethreutini (*E. tyrius)* has an ocellus. Enarmoniini, three Grapholitini (*Grapholita sp., C. pomonella,* and *D. vancouverana*), and all Eucosmini, except *Rhyacionia buoliana*, have a forewing ocellus.

The CuA1, M2, and M3 veins on the hind wing vary in their spatial relationship to each other (**character 27**). These three veins can be separate from each other, connate (fused at the base, originating from the same point), or CuA1+M3 can be connate or stalked with M2 separate in both conditions. CuA1 or M3 can be absent, and finally

M2+M3 can be stalked (present only in Tineidae). M3 is absent only in Gelechiidae. The remaining outgroups have CuA1, M2, and M3 separate (Acrolophidae, Yponomeutidae, and Urodidae) or CuA1+M3 connate and M2 separate (Oecophoridae and Galacticidae). All Chlidanotinae have CuA1+M3 connate and M2 separate. This condition present in 12 of the 25 species of Tortricinae and found in every tribe except Epitymbiini (all connate), and Shoenotenini (all separate) and includes: *P. asperana* (Phricanthini), *A. nephrodes* (Atterini), *A. humerosana* (Sparganothini), Ceracini, *D. histrionana* (Archipini), *A. alisellana* (Archipini), *C. alticolana* (Cnephasiini), *T. viridana* (Tortricini), *Orthocomotis sp.* (Cochylini [Euliini]), *E. ministrana* (Cochylini [Euliini]), *P. orphnoxantha* (Cochylini [Euliini]) and *E. busckana* (Cochylini). Three Tortricinae have CuA1+M3 stalked and M2 separate: *N. egens* (Cochylini [Euliini]), *A. variana* (Tortricini), and *P. flexilineana* (Phricanthini). *Decodes basiplagana* (Cnephasiini) has lost the CuA1 vein. Finally, in addition to Shoenotenini, both *Choristoneura* spp. (Archipini) and *A. rubigana* (Cochylini) have all veins separate. Within the Olethreutinae, all Eucosmini have CuA1+M3 stalked and M2 separate; this state is also found in *D. vancouverana* (Grapholitini), *A. nubeculana* (Enarmoniini), and *E. tyrius* (Olethreutini). Unlike the Tortricinae, the condition of CuA1+M3 connate and M2 separate is less common, being present only in Microcorsini, three Olethreutini (*P. niveiguttana, G. idia* [Gatesclarkeanini], and *E. hebesana* [Endotheniini]), and four Grapholitini (*L. subsolana, C. illepida, C. pomonella,* and *Grapholita sp.*). *B. maiorina* (Olethreutini [Bactrini]) has all veins connate, and *A. myrtillana* is missing CuA1. The following Olethreutini have all veins separate: *Pseudosciaphila duplex, Paralobesia cyclopiana, H. separatana, H.*

dimidana, L. aeolopa, and *O. fasciatana*). This character could not be coded for *E. mexicana* (Cochylini), *Platynota idaeusalis* (Sparganothini), and Glyphidoceridae.

Abdomen

Abdominal segment III of male Phricanthini (**node 8**) has a row of modified setae (**character 32**), which is absent in all other taxa sampled.

Scale sockets on the ventral surface of the abdomen are arranged randomly or in rows (**character 50**), both of which are present in the outgroups, and all three subfamilies. Within the Tortricinae, Phricanthini (**node 8**) have scale sockets in rows, whereas all other Tortricinae (**node 9**) have scales arranged randomly. Within the Chlidanotinae, Polyorthini, Hilarographini, and *A. dispersa* (Chlidanotini) have sockets arranged in rows, with other Chlidanotini (*T. climacias* and *H. tricesimana*) having scales arranged randomly. Many Olethreutinae have scales arranged in rows, including eight Olethreutini (*E tyrius, P. cyclopiana, P. niveiguttana, H. separatana, L. aeolopa, O. fasciatana, B. maiorina* [Bactrini], and *E. hebesana* [Endotheniini]), one Grapholitini (*L. subsolana*) and nine Eucosmini (*G. deludana, Epinotia infuscana, E. nisella, Crocidosema unica, Rhopobota naevana, Pseudexentera cressoniana,* and *P. costomaculana*). All other Olethreutinae have random scale socket arrangement. This character could not be seen for five Eucosmini (*Epinotia trigonella, Epiblema scudderiana, Gypsonoma sp., Eucosma radiatana, Pelochrista matutina*) and *A. nubeculana* (Enarmoniini).

Genitalia

With the exception of Microcorsini, all Olethreutinae (**node 22**) females have single-branched anterior apophyses that do not connect with the sterigma (**character 28**). All other taxa have two-branched anterior apophyses that connect to the sterigma by a sclerotized band. This character could not be seen for *P. orphnoxantha* (Cochylini [Euliini]) and *A. dispersa* (Atterini), however Horak and Brown (1991) indicate that all tortricines possess anterior apophyses with two branches.

A transtilla (**character 29**) is present in the male genitalia of all Chlidanotinae, Tortricinae and several of the outgroups (Yponomeutidae, Oecophoridae, and Galacticidae); however, it is missing in all Olethreutinae, Acrolophidae, and Glyphidoceridae. This character could not be seen for Urodidae, Gelechiidae, and Tineidae.

The socii of the male genitalia vary in their shape and attachment to the tegumen (**character 30**). Socii can be broadly connected laterally or dorsolaterally to the tegumen (usually laterally attached), narrowly connected laterally or dorsolaterally to the tegumen, ventrally pendant from top of tegumen, posteriorly projecting from top of tegumen, and finally, socii can be reduced or absent completely. Absence, or reduction, of socii is found in some outgroups and throughout most the tribes including: Urodidae, Galacticidae, Glyphidoceridae, Chlidanotinae (Chlidanotini*: T. climacias*), Tortricinae (Archipini: *D. histrionana, A. alisellana, C. rosaceana*; Epitymbiini; Cochylini: *E. ministrana* [Euliini]), and Olethreutinae (Microcorsini, and four Grapholitini: *D. vancouverana, C. illepida, C. pomonella, Grapholita sp.*). The remaining Chlidanotinae have either socii broadly connected to the tegumen, such as *P. episdesma* (Polyorthini)

and *H. tricesimana* (Chlidanotini), or socii that are ventrally pendant as in *T. jonesi* (Hilarographini). In Tortricinae socii with a narrow connection to the tegumen (broadening apically, usually broadest near the middle) are present in Phricanthini, Ceracini, *C. pinus* (Archipini), Cnephasiini, and all [Euliini] except *E. ministrana*. Three Cochylini (**node 21;** *A. rubigana, E. busckana*, and *E. mexicana*) have unique socii that that project posteriorly from the top of the tegumen (and are slender and with a uniform width). Sparganothini+Atterini (**node 11**) and Tortricini have socii that are broadly connected to the tegumen (usually laterally). This characteristic is shared by several outgroups (Acrolophidae, Yponomeutidae, and Oecophoridae) and many Olethreutinae taxa, including all Olethreutini, Enarmoniini, and 12 species of Eucosmini (*G. deludana, Epinotia infuscana, E. transmissana, E. solicitana, E. nisella, E. johnsonana, E. medioplagata, E. trigonella*, *Crocidosema unica, R. naevana, Gypsonoma sp.,* and *Catastega aceriella*). The remaining Olethreutinae have either absent or reduced socii (Microcorsini, and four Grapholitini: *D. vancouverana, C. illepida, C. pomonella, Grapholita sp.*) or ventrally pendant socii hanging from the top of the tegumen, which are present in *L. subsolana* (Grapholitini) and eight species of Eucosmini (*Pelochrista matutina, Eucopina tocullionana, Eucosma radiatana, Rhyacionia buoliana, S. ocellana, Pseudexentera cressoniana, P. costomaculana,* and *Epiblema scudderiana).* This character could not be seen in Tineidae, Gelechiidae, *A. dispersa* (Chlidanotini), Shoenotenini, and two Grapholitini (*L. subsolana,* and *G. idia* [Gatesclarkeanini])

All Olethreutinae have the juxta and phallus of the male genitalia fused (**character 31**), whereas it is articulated in Tortricinae, Chlidanotinae, and all outgroups.

A saccus of the male genitalia is present in the outgroups Urodidae, Gelechiidae, and Yponomeutidae. The only Tortricidae with a saccus (**character 33**) is Phricanthini.

The henion (**character 36**) of the male genitalia is unique to 10 species of Eucosmini (**node 31**; *G. deludana, Epinotia infuscana, E. solicitana, E. transmissana, E. nisella, E. johnsonana, E. medioplagata, E. trigonella, Crocidosema unica,* and *Catastega aceriella*). This character is absent in all other Tortricidae and outgroups.

A clasper (**character 37**) is present, on the valva of male genitalia, in five taxa sampled (one Tortricinae, four Olethreutinae), including *P. orphnoxantha* (Cochylini [Euliini]), *L. subsolana* (Grapholitini), and three Eucosmini (*R. naevana, Gypsonoma sp.,* and *Epiblema scudderiana*). All other taxa, including outgroups, lack a clasper.

Four species of Eucosmini (Olethreutinae) have saber-like socii, projecting dorsally (**character 38**); these include *Epinotia infuscana, E. solicitana*, and *Crocidosema unica* (**node 32**). All other taxa sampled lack saber-like socii.

The only known morphological apomorphy for Tortricidae are the large, flat, papillae anales (or 'ovipositor lobes') of the female genitalia (**character 39**) (Dugdale, 1988; Horak & Brown, 1991), and this state is present in all taxa sampled, excluding the eight outgroups.

The male genitalia of all Chlidanotinae sampled have valvae with an invagination (**character 41**) which holds a hair pencil (Horak & Brown, 1991). This character is absent in all other taxa.

The uncus of the male genitalia has a brush of setae on the ventral surface (**character 42**) in Archipini+Epitymbiini+Ceracini (**node 14**), but is absent in all other

sampled taxa. However, this character could not be seen in *T. climacias* (Chlidanotinae: Chlidanotini), *P. epidesma* (Chlidanotinae: Polyorthini), Gelechiidae, and Oecophoridae.

An apomorphy of Atterini is the presence of corethrogyne (**character 43**) on the abdomen of females, which are modified scales used to erect a fence around eggs (Horak & Brown, 1991). This trait is not present in any other taxa sampled.

Large, broad, densely scale socii (**character 44**) of the male genitalia are a trait unique to six members of the Tortricinae, including Sparganothini+Atterini (four species, **node 11**) and two species of Cochylini [Euliini] (*Orthocomotis sp.* and *B. salubricola*). All other taxa sampled lack this character state of socii.

Thought to be a unique apomorphy of the Cnephasiini, (Horak & Brown, 1991) the uncus of the male genitalia which is spinulose laterally (**character 45**) is not only present in Cnephasiini, but also *Heppnerographa tricesimana* (Chlidanotinae: Chlidanotini). No other taxa sampled possess this feature.

Also thought to be an apomorphy of Cnephasiini (Horak & Brown, 1991), presence of floricomous papillae anales (**character 46**) is also present in *T. viridana* (Tortricini); however, the papillae anales in *T. viridana* face posteriorly as opposed to ventrally in Cnephasiini and appear to have more depth (thicker) than Cnephasiini papillae anales. This character state is found in no other taxa sampled.

An enlarged juxta with a medially folded plate (**character 47**) is unique to the Tortricini and absent in all other taxa sampled.

Considered to be diagnostically important for Tortricini (Horak & Brown, 1991), presence of a subscaphium (sclerotized ventral area of anal tube, **character 48**) is not

only in both members of Tortricini, but also in *D. basiplagana* (Cnephasiini). No other taxa sampled have a subscaphium.

A gnathos (**character 51**) is present in the male genitalia of most Tortricidae, however it is absent in five Tortricinae (Cochylini: *E. mexicana, E busckana, A rubigana*), and one Chlidanotinae (Chlidanotini: *T. climacias*). This character is present in all other taxa except those that it could not be seen in (Grapholitini: *C. illepida, D. vancouverana;* Enarmoniini; Olethreutini: *E. hebesana* [Endotheniini], *B. maiorina* [Bactrini]; Microcorsini*: Cryptaspasma haplophyes;* Shoenotenini; Hilarographini; Acrolophidae, Tineidae, Glyphidoceridae, and Gelechiidae).

The signum of the female genitalia in Cnephasiini and *T. viridana* (Tortricini) is in the form of a dentate band, rarely stellate (Horak & Brown, 1991). This type of signa is absent from all other taxa (other forms of signa are present). This character could not be seen in *A. variana* (Tortricini).

Discussion and Conclusion

Endo- and exoskeletal characters

No new synapomorphies were found for any of the tribes, however, many tribes are united by or associated with other tribes by shared homoplasious apomorphies. Several non-traditional characters show clear trends consistent with previously known classification, with scale socket arrangement showing promising taxonomic utility for differentiating the tribes. For example, the clade represented by Archipini+Epitymbiini+Ceracini+Shoenotenini+Sparganothini+Atterini (**node 10**) share the same scale socket arrangement, 'random', on the procoxa (**character 7**), profemur (**character 8**), tegulae (**character 12)**, mesofemur (**character 14**), and the metafemur

(**character 17**). The first four characters (7, 8, 12, 14) distinguish this clade from all but one other tribe in the Tortricinae in which all or some members have scale sockets arranged in rows; the exception being Cnephasiini which has the same 'random' scale socket arrangement for the pro- and mesofemur (characters 7 and 14). Scale socket arrangement on the metafemur (**character 17**) is the same ('random') for all Tortricinae except Phricanthini ('rows'). Moreover, these four characters (7, 8, 12, 14) distinguish the aforementioned clade (**node 10**) from the Olethreutinae, which predominantly have scale sockets arranged in rows for the procoxa, profemur, tegulae, and mesofemur. Scale sockets of the tegulae are arranged in rows for nearly all Olethreutinae, except Enarmoniini, one Olethreutini (*E. tyrius*) and two Eucosmini (*Epinotia solicitana*, and *Rhyacionia buoliana*). Additionally, Enarmoniini has scale sockets arranged in elongate groups on the mesotibia, a trait shared only with [Bactrini] (Olethreutini), and seven Eucosmini, whereas all other Olethreutinae have scale sockets arranged in round groups. Although no apomorphy exists for all Enarmoniini, the tribe has several derived characters present only in this tribe (Horak, 2006); these are yet more characteristics that distinguish the Enarmoniini from nearly all other Olethreutinae. Two tribes of Chlidanotinae, Polyorthini and Hilarographini, consistently have scale sockets arranged in rows on the procoxa, profemur, pro-tarsomere I (**character 10**), tegulae, mesofemur, mesotibia, metafemur (only Polyorthini), metatibia, metatarsomere I, and ventral surface of the abdomen. Within the Chlidanotini, only the protarsomere I, metafemur, metatibia, and metatarsomere I, are arranged similarly for all taxa (with *A. dispersa* not being coded for the latter three due to missing the hind legs). All Tortricinae, except Phricanthini, have scale sockets arranged randomly on the ventral surface of the abdomen (**character**

50), whereas the outgroup, and all but two tortricid tribes (Microcorsini and Enarmoniini) have representatives with scale sockets arranged in rows. Unlike any other species, the sole representative of Ceracini, *C. xanthocosma*, has the same arrangement of scale sockets *for all eleven structures* examined (random in all).

Some characters show clear trends that distinguish the subfamilies; scales in the medial area of the frons (**character 1**) are present in all outgroups, and the majority of Chlidanotinae and Tortricinae, whereas Olethreutine has only four representatives with scales in the medial area (Microcorsini: *C. haplophyes;* Grapholitini: *Grapholita sp.* And *Cryptophlebia illepida;* Eucosmini: *S. ocellana*). Nearly all Olethreutinae (exceptions being two Eucosmini: *Rhyacionia buoliana* and *Gypsonoma sp.*) have an apophysis on the anterodorsal margin of the propleuron (**character 6**) whereas the majority of the Tortricinae (17/25 species) and Chlidanotinae (3/5 species) do not have an apophysis; those that do, appear to have a different form, being more squared and projecting posteriorly, however this was not distinguished from the Olethreutinae apophysis in the character matrix. None of the Olethreutinae or Chlidanotinae possess five or more spiniform setae on the protarsomeres (**character 11**), which is only present in Yponomeutidae, Gelechiidae, Oecophoridae, and in five Tortricinae representing Ceracini, Archipini, Cnephasiini, and [Euliini] (Cochylini). Furthermore, all Chlidanotinae with the exception of *H. tricesimana* (Chlidanotini), which has three, have either reduced spiniform setae or they are absent from the protarsomere. As with the protarsomeres, no Olethreutinae have five or more spiniform setae on the metatarsomeres (**character 20**), and only one Chlidanotinae has five or more (Chlidanotini: *H. tricesimana*); alternatively, five or more setae are found throughout the Tortricinae. The

proleg claw (**character 21**) is simple in all Olethreutinae and all but one Chlidanotinae, but is basally covered with microtrichia in the majority of Tortricinae (18/25 species), one Chlidanotinae (*H. tricesimana*) and in Oecophoridae. Although both Olethreutinae and Tortricinae have representatives from nearly all tribes with microtrichia on the metascutellum (**character 23**), none of the Olethreutinae have 'specialized' microtrichia (**character 35**) which are found only in three Tortricinae representing [Euliini] (Cochylini), Cnephasiini, and Shoenotenini, as well as *H. tricesimana* (Chlidanotinae: Chlidanotini). All Olethreutinae have scales on the metascutellum (**character 24**), whereas five Tortricinae taxa representing [Euliini] (Cochylini), Cnephasiini, Schoenotenini, and Atterini, have no scales on the metascutellum, a trait shared with only one *Heppnerographa tricesimana* (Chlidanotinae: Chlidanotini) and Oecophoridae.

A noticeable trend, and one reflected by the inclusion of the aforementioned *H. tricesimana* (Chlidanotinae) with the Tortricinae in Fig. 3.1, is that *H. tricesimana* shares many characteristics with the Tortricinae, specifically the Cnephasiini. Regardless of how characters are weighted (Compare Fig. 3.1 and 3.2) *H. tricesimana* is placed within the Tortricinae, as sister group to the Cnephasiini, even in analyses where characters were heavily weighted in favor of grouping Chlidanotinae together (by giving a weight of '4' to characters that unite the Chlidanotinae, and Chlidanotini). *Heppnerographa tricesimana* shares nine characters with Cnephasiini including: Protarsomere with three spiniform setae (all other Chlidanotinae have none or are reduced, **character 11**), tegulae with random scale arrangement (rows in all other Chlidanotinae, **character 12**), presence of a spiniform process on the furca (absent in all other Chlidanotinae, **character 13**), metatarsomere with five or more spiniform setae (three of fewer in all other

Chlidanotinae, **character 20**), proleg claw microtrichiate (simple in all other Chlidanotinae, **character 21** weighted at '2' in Fig. 3.1), metascutellum with microtrichia (absent in all other Chlidanotinae, **character 23**), metascutellum without scales (present in all other Chlidanotinae, **character 24**), metascutellum with specialized microtrichia (absent in all other Chlidanotinae, **character 35**) which are only present in three other taxa, and finally, an uncus that is spinulose laterally (absent in all other Chlidanotinae, **character 45** weighted at '2' in Fig. 3.1), considered to be a synapomorphy for Cnephasiini (Horak & Brown, 1991).

Agreement and disagreement with molecular-based phylogeny

Morphology (Fig. 3.1) supports the Chlidanotinae as being a monophyletic group, excluding *H. tricesimana* (Chlidanotini), which is a sister group of Cnephasiini. In contrast, Regier et al. (2012) has strong support for Polyorthini being basal to Hilarographini+Chlidanotini. Furthermore, the inclusion of *H. tricesimana* as sister group to *A. dispersa* (Chlidanotini) is well supported by Regier et al. (2012) and Fagua et al. (2016).

Phricanthes was originally included in the Olethreutinae based on morphology, but Meyrick (1910) transferred the genus to Tortricinae. Horak and Brown (1991) considered Phricanthini to be basal of all Tortricidae due to morphology and biology that includes their exclusive association with archaic Dilleniaceae, even though the tribe was retained in Tortricinae by default. The morphological analyses of endo- and exoskeletal characters (Fig. 3.1) supports the basal relationship to all other Tortricidae. In contrast, molecular data supports a sister group relationship with only Tortricinae (Fagua et al., 2016; Regier et al., 2012).

Tortricini is not included with the Tortricinae regardless of character weighting in the morphological tree. Kuznetsov and Stekolnikov (1984) included the tribe with the Chlidanotinae, based on genital musculature. With weighted characters (Fig. 3.1) Tortricini are placed as a sister group to Tortricinae (excluding Phricanthini) and Olethreutinae. With characters given equal weights (Fig. 3.2.), Tortricini are placed in a polytomy with Phricanthini, Chlidanotinae (excluding *H. tricesimana*), and Olethreutinae. This contradicts both Regier et al. (2012) and Fagua et al. (2016), which have strong support for Tortricini as being a sister group to the Cnephasiini. Certain morphological characters not previously considered synapomorphies support this grouping, including: stellate signum in the female genitalia of both tribes (**character 52**), floricomous papillae anales in the female genitalia of all Cnephasiini and periodically in the Tortricini (**character 46**), and the ventral part of anal tube sclerotized as a 'subscaphium' (**character 48**) in Tortricini and some Cnephasiini.

To the exclusion of Phricanthini, Regier et al. (2012) divided the Tortricinae into two monophyletic clades, the first being Archipini+Ceracini+Sparganothini+Atterini and the second that is Cochylini+Euliini+Tortricini+Cnephasiini. This contradicts both the analyses with 14 weighted characters (Fig. 3.1) and with all characters equally weighted (Fig. 3.2); however, in the weighted analysis, Archipini+Epitymbiini+Ceracini are monophyletic which is also true in the phylogeny produced by Fagua et al. (2016).

The morphological analysis places Epitymbiini within a monophyletic clade with Archipini+Ceracini, which is consistent with Fagua et al. (2016). Regier et al. (2012) provides strong support for Archipini+Ceracini, but did not include Epitymbiini in their analysis

Sparganothini+Atterini are well supported in both molecular studies (Fagua et al., 2016; Regier et al., 2012). This relationship is also supported by morphology (Fig. 3.1).

The Cochylini+[Euliini] demonstrate close relationships in both morphological trees (Figs. 3.1 and 3.2), however two of the five species formerly assigned to Euliini, *Bonagota salubricola* and *Orthocomotis sp.,* are allied to Sparganothini+Atterini in the weighted analysis (Fig. 3.1). This relationship is likely due to sharing a homoplasious apomorphy, weighted at '2' (**character 44**: socii with large scaled lobes), which is shared only with the Sparganothini+Atterini.

Within the Olethreutinae, Regier et al. (2012) have the Microcorsini as being the most basal tribe (100% bootstrap support), which is also demonstrated by Fagua et al. (2016) and consistent with the morphological analyses. Regier et al. (2012) and Fagua et al. (2016) have the remaining Olethreutinae divided into two monophyletic clades, both weakly supported (<50% bootstrap each in Regier et al., and <0.61 posterior probability in Fagua et al.); no such trend is demonstrated by the morphological analyses. Rather, the Grapholitini are basal to Eucosmini, Olethreutini, and Enarmoniini with one exception, *Larisa subsolana* (Grapholitini), which is included within the Olethreutini and sister group to *O. fasciatana.* Regier et al. (2012) and Fagua et al. (2016) did not include *L. subsolana* in their analyses, and all Grapholitini were included in a well supported monophyletic clade, sister group to the Eucosmini. The weighted and non-weighted morphological analyses divided Eucosmini into two clades with Enarmoniini and *Episimus tyrius* (Olethreutini) being included within these two clades in both cases. Regier et al. (2012) weakly supports (<50%) Enarmoniini as being basal to Eucosmini+Grapholitini whereas Fagua et al. (2016) provides strong support for

Enarmoniini being a sister group to Olethreutini, and include *E. tyrius* (Olethreutini) as within the Enarmoniini, however they make no formal suggestion for it's reclassification. The morphological analyses unite the Olethreutini in one monophyletic clade that includes [Gatesclarkeanini]+[Endothenini]+[Bactrini]; as mentioned, both analyses include *L. subsolana* (Grapholitini) as being within the Olethreutini.

Conclusions

Scale socket arrangement shows promise in defining tribes, and possibly species. The character is best seen when structures have high quality staining and are viewed at low magnifications (50x) on a compound scope. Staining with both Eosin Y and Chlorazol provides enough contrast between sclerotized structures and membranous regions of the socket to be able to easily view the scale socket arrangement. Viewing at high magnification can lead to misidentification of socket arrangements. Scales arranged in rows are usually obvious at low magnification, but these can appear random at higher magnifications can easily appear as random unless it's distinctive. Weak staining on old slides, prepared before enhanced staining had been investigated, resulted in an inability to code scale arrangement accurately for certain species, such as *Epinotia infuscana.*

Microtrichia on the metascutellum may be misrepresented in this study; upon discovering that males tended to have fewer, smaller, microtrichia than females, it became clear that species represented only be males may have been coded as not having microtrichia, when the female may possess this trait. This character would require both sexes to be represented in order to get an accurate estimate of it's distribution across the tribes. Furthermore, Common (1969) suggested that the microtrichia on the metascutum were for locking the wings to the thorax when at rest; if microtrichia on the

metascutellum have the same purpose, it can be postulated that females would be less likely to lose microtrichia as they are predominantly resting, whereas males are typically the active sex, and may have less 'need' for a wing locking mechanism.

Broader taxon sampling, with at least two species representing each tribe would strengthen the morphological analyses, particularly for the Tortricinae, where both Schoenotenini and Ceracini are represented by only one species. Additionally, more accurate conclusions could be made about the tribe with more taxa; for example, *C. xanthocosma* (Ceracini) was unique in being the only species to have a 'random' scale socket arrangement for all eleven structures coded for socket arrangement. It would be valuable to know if this is a synapomorphy for the tribe.

A principle components analysis would determine if certain characters are highly correlated with each other. Scale socket arrangement on the mesotarsomere I was identical to that of metatarsomere I and was thus excluded from the analysis. It's possible that other, more complicated, trends exist within morphological characters.

Table 3.1 List of Tortricidae used for whole body and genitalic preparations.

Subfamily	Tribe	Species	Whole	Genitalia
Chlidanotinae	Chlidanotini	* *Auratonota dispersa* Brown, 1990	1 (1M)	
		**Heppnerographa tricesimana* (Zeller, 1877)	1 (1M)	1 (1F)
		Trymalitis climacias Meyrick, 1911	1 (1F)	2 (1M,1F)
	Hilarographini	*Thaumatographa jonesi* (Brower, 1953)	1 (1M)	
	Polyorthini	*Polylopha epidesma* Lower, 1901	1 (1M)	2 (1M,1F)
Olethreutinae	[Bactrini]	* *Bactra maiorina* Heinrich, 1923	1 (1M)	2 (2F)
	Enarmoniini	* *Ancylis myrtillana* (Treitschke, 1830)	1 (1M)	1 (1F)
		**Ancylis nubeculana* (Clemens, 1860)	1 (1F)	
	Eucosmini	*Catastega aceriella* Clemens, 1861	2 (1M,1F)	
		Crocidosema unica (Heinrich, 1923)	1 (1M)	
		**Epiblema scudderiana* (Clemens, 1860)	1 (1M)	2 (2F)
		**Epinotia infuscana* (Walsingham, 1879)	1 (1M)	1 (1F)
		**Epinotia johnsonana* (Kearfott, 1907)	2 (2M)	
		**Epinotia medioplagata* (Walsingham, 1895)	1 (1M)	
		**Epinotia nisella* (Clerck, 1759)	3 (2M,1F)	
		**Epinotia solicitana* (Walker, 1863)	3 (3M)	2 (2F)
		**Epinotia transmissana* (Walker, 1863)	1 (1M)	1 (1F)
		**Epinotia trigonella* (Linnaeus, 1758)	1 (1M)	
		Eucopina tocullionana (Heinrich, 1920)	1 (1M)	2 (2F)
		Eucosma radiatana (Walsingham, 1879)	1 (1M)	
		Gretchena deludana (Clemens, 1864)	1 (1F)	2 (2F)
		**Gypsonoma* sp.	1 (1M)	1 (1F)
		**Pelochrista matutina* (Grote, 1873)	1 (1F)	1 (1F)
		Pseudexentera costomaculana (Clemens, 1860)	1 (1F)	2 (1M,1F)
		Pseudexentera cressoniana (Clemens, 1864)	1 (1F)	2 (1M,1F)
		Rhopobota naevana (Hübner, [1814-1817])	2 (2M)	2 (1M,1F)
		Rhyacionia buoliana (D & S)], 1775)	1 (1F)	1 (1F)
		**Spilonota ocellana* (D & S, 1775)	1 (1F)	
	† [Gatesclarkeanini]	*Gatesclarkeana idia* Diakonoff, 1973	1 (1F)	
	Grapholitini	**Cryptophlebia illepida* (Butler, 1882)	1 (1M)	
		**Cydia pomonella* (Linnaeus, 1758)	1 (1M)	
		**Dichrorampha vancouverana* McDunnough, 1935	1 (1M)	
		**Grapholita* sp.	1 (1F)	
		Larisa subsolana Miller, 1978	2 (2M)	
	Microcorsini	* *Cryptaspasma bipenicilla* Brown & Brown, 2004	1 (1M)	2 (1M,1F)
		**Cryptaspasma haplophyes* Diakonoff, 1959	1 (1M)	2 (1M,1F)
	[Endotheniini]	* *Endothenia hebesana* (Walker, 1863)	1 (1M)	2 (1M,1F)
	Olethreutini	* *Episimus tyrius* Heinrich, 1923	2 (1M,1F)	1 (1M)
		**Hedya dimidiana* (Clerck, 1759)	1 (1M)	1 (1F)
		**Hedya separatana* (Kearfott, 1907)	1 (1M)	1 (1F)

Table 3.1 (Continued)

		**Lobesia aeolopa* Meyrick, 1907	1 (1M)	
		**Olethreutes fasciatana* (Clemens, 1860)	1 (1M)	2 (1M,1F)
		Paralobesia cyclopiana (Heinrich, 1926)	1 (1M)	
		Phaecasiophora niveiguttana (Grote, 1873)	1 (1M)	
		Pseudosciaphila duplex (Walsingham, 1905)	1 (1M)	
Tortricinae	Archipini	**Argyrotaenia alisellana (Robinson, 1869)*	1 (1M)	1 (1F)
		**Choristoneura pinus* Freeman, 1953	1 (1M)	2 (1M,1F)
		**Choristoneura rosaceana* (Harris, 1841)	2 (1M,1F)	
		**Dichelia histrionana* (Frolich, 1828)	1 (1M)	1 (1F)
	Atterini	**Anacrusis nephrodes* (Walsingham, 1914)	1 (1M)	
		Tinacrucis sp.	1 (1F)	
	Ceracini	**Cerace xanthocosma* Diakonoff, 1950	1 (1M)	1 (1F)
	Cnephasiini	** Cnephasia alticolana* (Herrich-Schäffer, 1851)	1 (1M)	1 (1F)
		**Decodes basiplagana* (Walsingham, 1879)	1 (1M)	2 (1M,1F)
	Cochylini	** Aethes rubigana* (Treitschke, 1830)	2 (1M,1F)	
		**Eugnosta busckana* (Comstock, 1939)	1 (1M)	1 (1F)
		**Eugnosta mexicana* (Busck, 1907)	1 (1M)	1 (1M)
	† Epitymbiini	*Anisogona mediana* (Walker, 1863)	1 (1M)	
	Euliini	** Netechma egens* Razowski, 1999	1 (1M)	
		**Bonagota salubricola* (Meyrick, 1931)	2 (1M,1F)	
		**Eulia ministrana* (Linnaeus, 1758)	1 (1M)	
		**Pseudomeritastis orphnoxantha* Obraztsov, 1966	1 (1M)	
		Orthocomotis sp.	2 (1M,1F)	
	Phricanthini	** Phricanthes asperana* Meyrick, 1881	1 (1M)	
		** Phricanthes flexilineana* (Walker, 1863)	1 (1M)	
	† Schoenotenini	*Palaeotoma styphelana* Meyrick, 1881	1 (1F)	
	Sparganothini	**Amorbia humerosana* Clemens, 1860	1 (1M)	3 (2M,1F)
		** Platynota idaeusalis* (Walker, 1859)	1 (1M)	1 (1F)
	Tortricini	**Acleris variana* (Fernald, in Packard, 1886)	1 (1M)	2 (2F)
		Tortrix viridana Linnaeus, 1758	1 (1M)	1 (1F)
Outgroup	Acrolophidae	*Acrolophus arcanella* (Clemens, 1859)	1 (1F)	1 (1F)
Outgroup	Galacticidae	*Homadaula anisocentra* Meyrick, 1922	2 (1M,1F)	
Outgroup	Gelechiidae	Dichomeris ligulella Hübner, 1818	1 (1F)	1 (1M)
Outgroup	Glyphidoceridae	Glyphidocera juniperella Adamski, 1987	1 (1M)	1 (1F)
Outgroup	Oecophoridae	Machimia tentoriferella Clemens, 1860	1 (1M)	1 (1F)
Outgroup	Tineidae	Setomorpha rutella Zeller, 1852	2 (1M,1F)	
Outgroup	Urodidae	Urodus parvula (Edwards, 1881)	1 (1M)	1 (1F)
Outgroup	Yponomeutidae	Yponomeuta multipunctella Clemens, 1860	1 (1M)	1 (1F)

Denis & Schiffermüller are abbreviated as "D &S". Genera examined by Regier et al. (2012) are marked with an asterisk '*' to the left of the name. Tribes not examined by Regier et al. (2012) are marked with a †

Table 3.2 List of character and character states

1	**Frons** with scales in medial area: 0=absent or sparse; 1=present.
2	**Frons** with scales in tract between antennal sulci: 0=absent; 1=present.
3	**Antennal socket** surrounded by microtrichia: 0=present; 1=absent.
4	**Sitophore** apical margin: 0=angled; 1=straight; 2=curved; 3=concave.
5	**Sitophore** sensilla: 0= 2 pairs posterior, 2 pairs anterior; 1=2 pairs posterior, 2 groups of 10 each anterior; 2=2 pair posterior, 2 groups of 5-7 each anterior; 3=2 pair posterior, 2 groups of three anterior; 4=1 pair posterior, 4 irregular anterior; 5=2 pairs posterior, 0 anterior; 6=2 single posterior, 2 single anterior.
6	**Propleuron** anterodorsal margin with apophysis: 0=forming apophysis; 1=absent.
7	**Procoxal** scale sockets: 0=random; 1=rows.
8	**Profemur** scale sockets: 0=random; 1=rows.
9	**Protibia** scale sockets: 0=random; 1=rows; 2=elongate groups; 3=round groups.
10	**Pro-tarsomere I** scale sockets: 0=random; 1=rows; 2=elongate groups.
11	**Pro-tarsomeres** with spiniform setae: 0=no spiniform setae or reduced; 1=3 spiniform setae; 2=2 spiniform setae; 3= 5 or more.
12	**Tegula** scale sockets: 0=random; 1=rows.
13	**Furca** with dentate projection on spiniform process: 0=present on process; 1=present at base of process; 2=absent.
14	**Mesofemur** scale sockets: 0=random; 1=rows.
15	**Mesotibia** scale sockets: 0=random; 1=rows; 2=elongate groups; 3=round groups.
16	**Euphragma** with lateral flanges: 0=present; 1=absent.
17	**Metafemur** scale sockets: 0=random; 1=rows.
18	**Metatibia** scale sockets: 0=random; 1=rows; 2=round groups.
19	**Meta-tarsomere I** scale sockets: 0=random; 1=round group.
20	**Meta-tarsomeres I-V:** 0=no spiniform setae or reduced; 1=3 spiniform setae; 2=4 spiniform setae; 3=5 or more.
21	**Proleg claw:** 0=simple; 1=microtrichiate.
22	**Ocellus:** 0=present; 1=absent.
23	**Metascutellum** with microtrichia: 0=absent; 1=present.
24	**Metascutellum** with scales: 0=present; 1= absent.
25	**Forewing** with tufted scales: 0=absent; 1=present.
26	**Forewing** with ocellus: 0=absent; 1=present.
27	**Hindwing** CuA1, M2, and M3: 0=separate; 1=all connate; 2=CuA1+M3 stalked, M2 separate; 3=M3+CuA1 connate, M2 separate; 4=CuA1 absent; 5=M3 absent; 6=M2+M3 stalked.
28	**Anterior apophyses:** 0=two branches; 1= one branch. Weighted at 2.
29	**Transtilla**: 0=present; 1=absent. Weighted at 2.
30	**Socii**: 0=Laterally; broadly connected to tegumen; 1=ventrally pendant (hanging from top of tegumen); 2=absent or reduced; 3=narrowly connected to tegumen; broadens apically then tapers (broadest in middle) 4=slender w/ uniform width; fingerlike projection off top of tegumen. Weighted at 2.
31	**Phallus and juxta:** 0=articulated; 1=fused. Weighted at 2.
32	**Abdominal segment III** of male with row of modified setae: 0=present; 1=absent.
33	**Saccus**: 0=present; 1=absent.

Table 3.2 (Continued)

34	**Vertex**, width of anterior scaled area and inter-antennal socket width ratio: 0=0.4; 1=0.5; 2=0.6; 3=0.7; 4=0.8; 5=0.9; 6=1.0.
35	**Metascutellum** with "velcro" specialized microtrichia: 0=absent; 1=present.
36	**Phallus** with henion: 0=absent; 1=present. Weighted at 2.
37	**Valva with clasper**: 0=absent; 1=present.
38	**Socii** saber like: 0=absent; 1=present. Weighted at 2.
39	**Papillae anales**: 0=various forms, not flat; 1=flat.
40	**Frons** lower scales: 0=directed ventrally; 1=directed dorsally.
41	**Valva** invaginated and holding hair pencil: 0=absent; 1=present. Weighted at 2.
42	**Uncus** with brush of setae on ventral surface: 0=absent; 1=present. Weighted at 2.
43	**Corethrogyne** in female: 0=absent; 1=present.
44	**Socii** with large scale lobes: 0=absent; 1=present. Weighted at 2.
45	**Uncus** spinulose laterally: 0=absent; 1=present. Weighted at 2.
46	**Papillae anales**: 0=not floricomous; 1=floricomous. Weighted at 2.
47	**Juxta**: 0=not enlarged and with medially folded plate; 1=enlarged and with medially folded plate.
48	**Subscaphium**: 0=not enlarged and sclerotized; 1=enlarged and sclerotized. Weighted at 2.
49	**Antennae** with distal flagellomeres compressed (scales appressed): 0=absent; 1=present.
50	**Abdomen** scale arrangement (ventral): 0=random; 1=rows.
51	**Gnathos:** 0=present; 1=absent. Weighted at 2.
52	**Signum** a dentate band or stellate: 0=absent; 1=present. Weighted at 2.

Table 3.3 Morphological data matrix for 52 characters in 82 taxa.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Acleris variana	1	0	1	2	2	0	1	1	2	2	0	1	1	1	3	0	0	2
Acrolophus arcanella	1	0	1	3	-	1	0	0	0	2	1	0	1	0	0	1	0	0
Aethes rubigana	0	0	1	3	5	1	1	1	2	2	1	1	0	1	2	0	0	2
Amorbia humerosana	0	0	1	1	2	0	0	0	2	2	1	0	1	0	2	0	0	2
Anacrusis nephrodes	1	0	0	1	1	1	0	0	2	2	1	0	1	0	1	0	0	2
Ancylis myrtillana	0	0	1	2	2	0	1	1	2	2	1	0	0	1	2	0	0	2
Ancylis nubeculana	0	0	1	2	-	0	1	1	2	2	1	0	1	1	2	0	0	2
Anisogona mediana	1	0	1	-	3	1	0	0	2	2	1	0	1	0	2	0	0	2
Argyrotaenia alisellana	1	0	1	1	0	1	0	0	2	2	1	0	1	0	2	0	0	2
Auratonota dispersa	0	0	1	1	2	1	1	1	3	2	0	1	2	1	2	0	-	-
Bactra maiorina	0	0	0	0	0	0	0	1	2	2	0	1	0	1	2	0	0	2
Bonagota salubricola	0	0	1	1	2	1	1	1	2	2	3	1	1	1	2	0	0	2
Catastega aceriella	0	0	1	2	1	0	0	1	2	2	1	1	1	1	3	0	0	2
Cerace xanthocosma	1	0	1	3	2	1	0	0	0	0	3	0	1	0	0	-	0	0
Choristoneura pinus	1	0	1	0	2	1	0	0	2	2	3	0	1	0	2	0	0	2
Choristoneura rosaceana	1	0	1	0	2	1	0	0	2	2	1	0	1	0	3	1	0	2
Cnephasia alticolana	0	0	1	2	2	1	0	0	2	2	3	0	1	0	2	0	0	2
Crocidosema unica	0	0	1	1	1	0	1	1	2	2	1	-	1	1	2	-	1	2
Cryptaspasma bipenicilla	0	0	1	2	2	0	0	1	2	2	0	1	1	1	3	0	1	2
Cryptaspasma haplophyes	1	0	1	-	-	0	1	1	2	2	0	1	1	1	3	0	1	2
Cryptophlebia illepida	1	0	1	1	1	0	1	1	2	2	1	1	1	1	3	0	0	0
Cydia pomonella	0	0	1	1	2	0	1	1	2	2	1	1	0	1	3	0	0	2
Decodes basiplagana	0	0	1	2	2	1	1	0	2	2	1	1	1	0	2	0	0	2
Dichelia histrionana	1	0	1	1	2	1	0	0	2	2	1	0	1	0	2	0	0	2
Dichomeris ligulella	1	0	1	3	6	0	1	1	1	2	3	1	2	1	1	1	1	1
Dichrorampha vancouverana	0	0	1	1	2	0	1	1	2	2	1	1	1	1	3	0	0	2
Endothenia hebesana	0	0	1	1	0	0	1	1	3	2	0	1	1	1	3	0	1	2
Epiblema scudderiana	0	1	1	0	-	0	1	1	2	2	1	1	1	1	3	0	0	2
Epinotia infuscana	0	0	1	-	2	0	1	1	-	-	1	1	1	1	3	0	1	2
Epinotia johnsonana	0	0	1	0	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Epinotia medioplagata	0	0	1	2	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Epinotia nisella (697)	0	0	1	0	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Epinotia nisella (698)	0	0	1	0	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Epinotia nisella (699)	0	0	1	0	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Epinotia solicitana (721)	0	0	1	1	2	0	0	0	2	2	1	0	1	0	3	-	0	2
Epinotia solicitana (722)	0	0	1	1	2	0	0	0	2	2	1	0	1	0	3	0	0	2
Epinotia solicitana (733)	0	0	1	1	2	-	0	0	2	2	1	0	1	0	2	0	0	2
Epinotia transmissana	0	1	1	1	2	0	1	1	2	2	1	1	1	1	2	0	0	2
Epinotia trigonella	0	1	1	0	1	0	0	0	2	2	1	1	1	-	-	0	0	2
Episimus tyrius	0	0	1	1	2	0	1	1	2	2	-	0	1	1	3	0	1	2

Table 3.3 (Continued)

Species	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Acleris variana	1	0	0	0	0	0	0	0	2	0	0	0	0	1	1	3	0	0
Acrolophus arcanella	1	1	0	1	0	0	1	0	0	0	1	0	0	1	1	4	0	0
Aethes rubigana	1	1	1	0	1	0	0	0	0	0	0	4	0	1	1	2	0	0
Amorbia humerosana	1	1	1	-	1	0	0	0	3	0	0	0	0	1	1	-	0	0
Anacrusis nephrodes	1	3	1	0	1	0	0	0	3	0	0	0	0	1	1	2	0	0
Ancylis myrtillana	1	1	0	0	1	0	0	1	4	1	1	0	1	1	1	2	0	0
Ancylis nubeculana	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	1	0	0
Anisogona mediana	1	1	0	0	0	0	0	0	1	0	0	2	0	1	1	-	0	0
Argyrotaenia alisellana	1	1	1	0	0	0	0	0	3	0	0	2	0	1	1	2	0	0
Auratonota dispersa	-		0	0	0	0	0	0	3	-	0	-	0	1	1	2	0	0
Bactra maiorina	1	0	0	0	1	0	0	0	1	1	1	0	1	1	1	1	0	0
Bonagota salubricola	1	3	1	0	1	0	0	0	2	0	0	3	0	1	1	3	1	0
Catastega aceriella	1	1	0	0	0	0	0	1	2	1	1	0	1	1	1	2	0	1
Cerace xanthocosma	0	3	0	0	0	0	0	0	3	0	0	3	0	1	1	3	0	0
Choristoneura pinus	1	3	1	0	0	0	0	0	0	0	0	3	0	1	1	4	0	0
Choristoneura rosaceana	1	3	1	0	1	0	0	0	0	0	0	2	0	1	1	5	0	0
Cnephasia alticolana	1	3	1	0	0	1	0	0	3	0	0	3	0	1	1	2	0	0
Crocidosema unica	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	0	0	1
Cryptaspasma bipenicilla	1	1	0	0	0	0	0	0	3	0	1	2	1	1	1	2	0	0
Cryptaspasma haplophyes	1	1	0	0	0	0	0	0	3	0	1	2	1	1	1	2	0	0
Cryptophlebia illepida	1	1	0	0	1	0	0	0	3	1	1	2	1	1	1	3	0	0
Cydia pomonella	1	1	0	0	1	0	0	1	3	1	1	2	1	1	1	1	0	0
Decodes basiplagana	1	2	1	0	1	1	1	0	4	0	0	3	0	1	1	3	1	0
Dichelia histrionana	1	2	1	0	0	0	0	0	3	0	0	2	0	1	1	2	0	0
Dichomeris ligulella	1	3	0	1	0	0	0	0	5	0	-	-	0	1	0	6	0	0
Dichrorampha vancouverana	1	1	0	0	0	0	0	1	2	1	1	2	1	1	1	4	0	0
Endothenia hebesana	1	0	0	0	0	0	0	0	3	1	1	0	1	1	1	1	0	0
Epiblema scudderiana	1	1	0	0	1	0	0	1	2	1	1	1	1	1	1	3	0	0
Epinotia infuscana	-	1	0	0	1	0	0	1	2	1	1	0	1	1	1	?	0	1
Epinotia johnsonana	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	2	0	1
Epinotia medioplagata	1	1	0	0	0	0	0	1	2	1	1	0	1	1	1	2	0	1
Epinotia nisella (697)	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	1	0	1
Epinotia nisella (698)	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	1	0	1
Epinotia nisella (699)	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	2	0	1
Epinotia solicitana (721)	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	1	0	1
Epinotia solicitana (722)	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	1	0	1
Epinotia solicitana (733)	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	-	0	1
Epinotia transmissana	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	3	0	1
Epinotia trigonella	1	1	0	0	0	0	0	1	2	1	1	0	1	1	1	3	0	1
Episimus tyrius	1	1	0	0	0	0	0	1	2	1	1	0	1	1	1	0	0	0

Table 3.3 (Continued)

Species	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
Acleris variana	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	-
Acrolophus arcanella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	0
Aethes rubigana	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Amorbia humerosana	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Anacrusis nephrodes	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0
Ancylis myrtillana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	-	0
Ancylis nubeculana	0	0	1	1	0	0	0	0	0	0	0	0	0	-	-	0
Anisogona mediana	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Argyrotaenia alisellana	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Auratonota dispersa	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0
Bactra maiorina	0	0	1	1	0	0	0	0	0	0	0	0	0	1	-	0
Bonagota salubricola	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Catastega aceriella	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Cerace xanthocosma	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Choristoneura pinus	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Choristoneura rosaceana	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Cnephasia alticolana	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1
Crocidosema unica	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Cryptaspasma bipenicilla	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Cryptaspasma haplophyes	0	0	1	1	0	0	0	0	0	0	0	0	0	0	-	0
Cryptophlebia illepida	0	0	1	1	0	0	0	0	0	0	0	0	0	0	-	0
Cydia pomonella	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Decodes basiplagana	0	0	1	1	0	0	0	0	1	1	0	1	0	0	0	1
Dichelia histrionana	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Dichomeris ligulella	0	0	0	0	0	-	0	0	0	0	0	0	0	1	-	0
Dichrorampha vancouverana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	-	0
Endothenia hebesana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	-	0
Epiblema scudderiana	1	0	1	1	0	0	0	0	0	0	0	0	0	-	0	0
Epinotia infuscana	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Epinotia johnsonana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Epinotia medioplagata	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Epinotia nisella (697)	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Epinotia nisella (698)	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Epinotia nisella (699)	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Epinotia solicitana (721)	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Epinotia solicitana (722)	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Epinotia solicitana (733)	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Epinotia transmissana	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Epinotia trigonella	0	0	1	1	0	0	0	0	0	0	0	0	0	-	0	0
Episimus tyrius	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0

Table 3.3 (Continued)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Eucopina tocullionana	0	0	1	1	2	0	0	1	2	2	1	-	1	1	3	0	0	2
Eucosma radiatana	0	-	1	1	2	0	0	0	2	2	1	1	1	-	-	0	0	2
Eugnosta busckana	1	0	1	1	2	1	0	0	2	2	1	0	0	0	2	0	0	2
Eugnosta mexicana	1	0	1	1	5	1	1	1	2	2	1	1	1	1	2	0	0	2
Eulia ministrana	1	0	1	2	2	1	1	0	2	2	1	1	1	0	2	0	0	2
Gatesclarkeana idia	0	0	1	1	2	0	1	1	3	2	0	1	1	1	3	0	0	2
Glyphidocera juniperella	1	0	1	2	6	1	1	1	2	2	2	1	1	1	2	1	0	0
Grapholita sp.	1	0	1	-	-	0	0	1	2	2	1	1	2	1	3	0	0	2
Gretchena deludana	0	0	1	1	2	0	1	1	2	2	1	1	1	1	2	0	0	2
Gypsonoma sp.	0	0	1	1	2	1	1	1	2	2	1	1	1	1	3	-	1	2
Hedya dimidiana	0	0	1	-	2	0	1	1	3	2	1	1	0	1	3	0	0	2
Hedya separatana	0	0	1	1	1	0	1	1	3	2	1	1	1	1	3	0	1	2
Heppnerographa tricesimana	1	0	1	2	2	1	1	0	2	2	1	0	1	0	2	0	0	2
Homadaula anisocentra	1	0	1	1	3	0	1	1	1	1	1	1	1	1	1	-	0	2
Larisa subsolana	0	0	1	1	3	0	1	1	2	2	1	1	0	1	3	0	0	2
Lobesia aeolopa	0	0	1	2	2	0	1	1	3	2	1	1	2	1	3	0	0	2
Machimia tentoriferella	1	0	1	2	6	1	-	0	2	2	3	1	2	0	0	1	0	0
Netechma egens	1	0	1	1	2	1	1	-	2	2	1	1	0	-	2	-	0	2
Olethreutes fasciatana	0	0	1	0	2	0	1	1	2	2	1	1	0	1	3	0	0	2
Orthocomotis sp.	1	0	1	1	2	0	0	1	2	2	3	1	1	1	2	0	0	2
Palaeotoma styphelana	0	0	1	2	3	1	0	0	2	2	1	0	1	0	2	0	0	2
Paralobesia cyclopiana	0	0	1	1	1	0	1	1	3	2	1	1	1	1	3	-	0	2
Pelochrista matutina	0	0	1	1	4	0	1	1	2	2	1	-	1	1	2	0	0	2
Phaecasiophora niveiguttana	0	0	1	2	2	0	1	1	1	2	1	1	1	1	3	0	0	2
Phricanthes asperana	1	0	0	1	2	0	1	1	2	2	0	1	0	1	2	0	1	2
Phricanthes flexilineana	1	0	0	1	2	0	1	1	2	2	0	1	0	1	3	0	1	2
Platynota idaeusalis	0	0	1	1	2	0	0	0	2	2	1	0	1	0	2	0	0	2
Polylopha epidesma	0	1	1	1	2	0	1	1	2	2	0	1	1	1	3	0	1	2
Pseudexentera costomaculana	0	1	1	0	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Pseudexentera cressoniana	0	0	1	2	2	0	1	1	2	2	1	1	1	1	3	0	1	2
Pseudomeritastis orphnoxantha	1	0	1	1	2	0	0	-	-	-	-	1	1	0	2	0	0	2
Pseudosciaphila duplex	0	0	1	1	2	0	0	1	2	2	1	1	1	1	3	0	0	2
Rhopobota naevana	-	0	1	1	1	0	1	1	2	2	1	1	1	1	3	0	1	2
Rhyacionia buoliana	0	0	1	1	3	1	0	0	2	2	1	0	1	0	2	0	0	2
Setomorpha rutella	1	0	1	1	-	1	1	1	0	0	0	1	2	1	1	1	0	2
Spilonota ocellana	1	0	1	2	2	0	0	0	2	2	1	-	1	0	2	0	0	2
Thaumatographa jonesi	1	0	1	2	2	0	1	1	2	2	0	1	2	1	3	0	0	2
Tinacrucis sp.	1	0	1	1	2	1	0	0	2	2	1	0	1	0	3	-	0	2
Tortrix viridana	1	0	1	1	2	0	1	1	2	2	0	1	1	1	3	0	0	2
Trymalitis climacias	1	0	1	1	2	1	0	0	0	2	0	1	2	0	3	1	0	2

Table 3.3 (Continued)

Species	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Eucopina tocullionana	1	1	0	0	0	0	0	1	2	1	1	1	1	1	1	1	0	0
Eucosma radiatana	1	1	0	0	1	0	0	1	2	1	1	1	1	1	1	2	0	0
Eugnosta busckana	1	1	1	0	0	0	0	0	3	0	0	4	0	1	1	4	0	0
Eugnosta mexicana	1	1	1	0	0	0	0	0	-	0	0	4	0	1	1	3	0	0
Eulia ministrana	1	2	1	0	0	0	0	0	3	0	0	2	0	1	1	1	0	0
Gatesclarkeana idia	1	0	0	0	1	0	0	0	3	1	-	-	1	1	1	0	0	-
Glyphidocera juniperella	1	1	0	1	0	0	0	-	-	0	1	2	0	1	1	-	0	0
Grapholita sp.	1	1	0	0	1	0	0	1	3	1	1	2	1	1	1	-	0	0
Gretchena deludana	1	1	0	0	1	0	0	1	2	1	1	0	1	1	1	1	0	1
Gypsonoma sp.	1	1	0	0	0	0	0	1	2	1	1	0	1	1	1	-	0	0
Hedya dimidiana	1	1	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0	0
Hedya separatana	1	1	0	0	0	0	0	0	0	1	1	0	1	1	1	3	0	0
Heppnerographa tricesimana	1	3	1	0	1	1	0	0	3	0	0	0	0	1	1	3	1	0
Homadaula anisocentra	1	-	0	1	0	0	0	0	3	0	0	2	0	1	-	2	0	0
Larisa subsolana	1	1	0	0	0	0	0	0	3	1	1	1	1	1	1	-	0	0
Lobesia aeolopa	1	1	0	0	1	0	0	0	0	1	1	-	1	1	1	3	0	0
Machimia tentoriferella	1	3	1	1	0	1	0	0	3	0	0	0	0	1	1	-	0	0
Netechma egens	1	1	1	0	1	0	0	0	2	0	0	3	0	1	1	3	0	0
Olethreutes fasciatana	1	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	0	0
Orthocomotis sp.	1	3	1	0	1	0	0	0	3	0	0	3	0	1	1	1	0	0
Palaeotoma styphelana	1	1	1	1	1	1	1	0	0	0	0	-	0	1	1	4	1	0
Paralobesia cyclopiana	1	1	0	0	1	0	0	0	0	1	1	0	1	1	1	-	0	0
Pelochrista matutina	1	1	0	0	1	0	0	1	2	-	1	1	1	1	1	1	0	0
Phaecasiophora niveiguttana	1	1	0	0	1	0	0	0	3	1	1	0	1	1	1	1	0	0
Phricanthes asperana	1	0	0	0	1	0	1	0	3	0	0	3	0	0	0	1	0	0
Phricanthes flexilineana	1	0	-	0	1	0	0	0	2	0	0	3	0	0	0	2	0	0
Platynota idaeusalis	1	1	0	0	0	0	0	0	-	0	0	0	0	1	1	-	0	0
Polylopha epidesma	1	0	0	0	0	0	1	0	3	0	0	0	0	1	1	3	0	0
Pseudexentera costomaculana	1	1	0	0	1	0	0	1	2	1	1	1	1	1	1	1	0	0
Pseudexentera cressoniana	1	1	0	0	1	0	0	1	2	1	1	1	1	1	1	-	0	0
Pseudomeritastis orphnoxantha	1	3	-	1	0	1	0	0	3	-	0	3	0	1	1	1	0	0
Pseudosciaphila duplex	1	1	0	0	1	0	0	0	0	1	1	0	1	1	1	0	0	0
Rhopobota naevana	1	1	0	0	0	0	0	1	2	1	1	0	1	1	1	-	0	0
Rhyacionia buoliana	1	1	0	0	0	0	0	0	2	1	1	1	1	1	1	-	0	0
Setomorpha rutella	1	0	0	1	0	0	0	0	6	0	-	-	0	1	-	5	0	0
Spilonota ocellana	1	1	0	0	1	0	0	1	2	1	1	1	1	1	1	3	0	0
Thaumatographa jonesi	1	1	0	0	0	0	0	0	3	0	0	1	0	1	1	0	0	0
Tinacrucis sp.	1	3	1	0	0	1	0	0	1	0	0	0	0	1	1	3	0	0
Tortrix viridana	1	0	1	0	1	0	0	0	3	0	0	0	0	1	1	2	0	0
Trymalitis climacias	1	0	0	0	0	0	0	0	3	0	0	2	0	1	1	3	0	0

Table 3.3 (Continued)

Species	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
Eucopina tocullionana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Eucosma radiatana	0	0	1	1	0	0	0	0	0	0	0	0	0	-	0	0
Eugnosta busckana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
Eugnosta mexicana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
Eulia ministrana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Gatesclarkeana idia	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Glyphidocera juniperella	0	0	0	0	0	-	0	0	0	0	0	0	0	1	-	0
Grapholita sp.	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Gretchena deludana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Gypsonoma sp.	1	0	1	1	0	0	0	0	0	0	0	0	0	-	0	0
Hedya dimidiana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Hedya separatana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Heppnerographa tricesimana	0	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0
Homadaula anisocentra	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larisa subsolana	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Lobesia aeolopa	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Machimia tentoriferella	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0
Netechma egens	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Olethreutes fasciatana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Orthocomotis sp.	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Palaeotoma styphelana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	-	0
Paralobesia cyclopiana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Pelochrista matutina	0	0	1	1	0	0	0	0	0	0	0	0	0	-	0	0
Phaecasiophora niveiguttana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Phricanthes asperana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Phricanthes flexilineana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Platynota idaeusalis	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
Polylopha epidesma	0	0	1	1	1	-	0	0	0	0	0	0	0	1	0	0
Pseudexentera costomaculana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Pseudexentera cressoniana	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Pseudomeritastis orphnoxantha	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Pseudosciaphila duplex	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Rhopobota naevana	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0
Rhyacionia buoliana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Setomorpha rutella	0	0	0	0	0	0	0	0	0	0	0	0	0	1	-	0
Spilonota ocellana	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Thaumatographa jonesi	0	0	1	1	1	0	0	0	0	0	0	0	0	1	-	0
Tinacrucis sp.	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0
Tortrix viridana	0	0	1	1	0	0	0	0	0	1	1	1	0	0	1	1
Trymalitis climacias	0	0	1	1	1	-	0	0	0	0	0	0	1	0	1	0

Table 3.3 (Continued)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Urodus parvula	1	0	1	1	6	1	1	0	0	2	-	1	2	0	0	1	0	0
Yponomeuta multipunctella	1	0	1	2	2	1	1	1	0	2	3	1	1	1	2	0	0	0

Species	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Urodus parvula	0	-	0	1	0	0	0	0	0	0	-	2	0	1	0	4	0	0
Yponomeuta multipunctella	0	3	0	1	0	0	0	0	0	0	0	0	0	1	0	5	0	0

Species	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
Urodus parvula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yponomeuta multipunctella	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

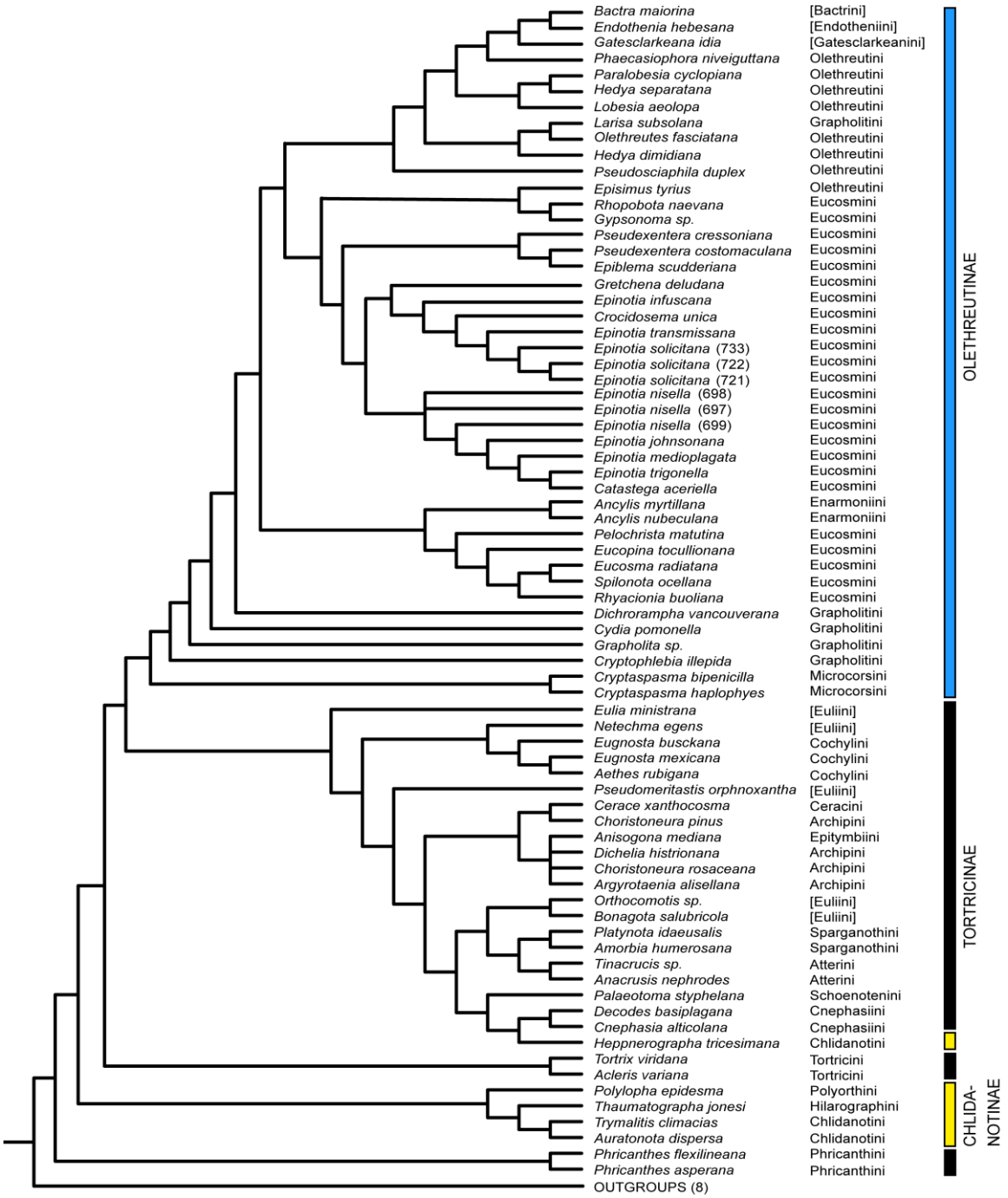

Figure 3.1 Strict consensus tree (length=392, CI=0.2551, RI=0.7021) of five equally most parsimonious trees (length=389, CI=0.2571, RI=0.7051) with 14 characters given a weight of '2'.

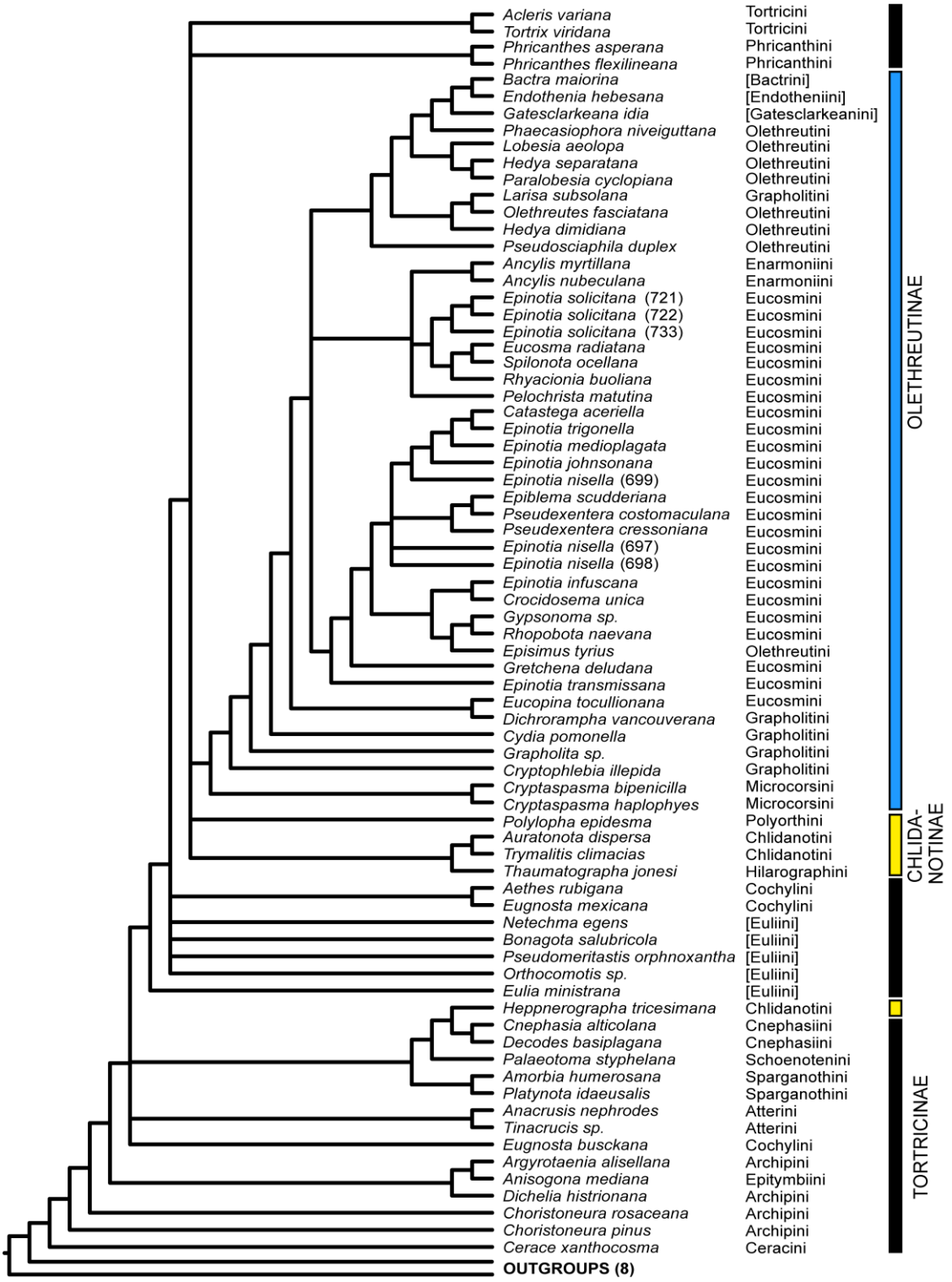

Figure 3.2 50% majority rule consensus tree (length=360, CI=0.2306, RI=0.6516) of six equally most parsimonious trees (length=345, CI=0.2406, RI=0.6704) with characters equally weighted.

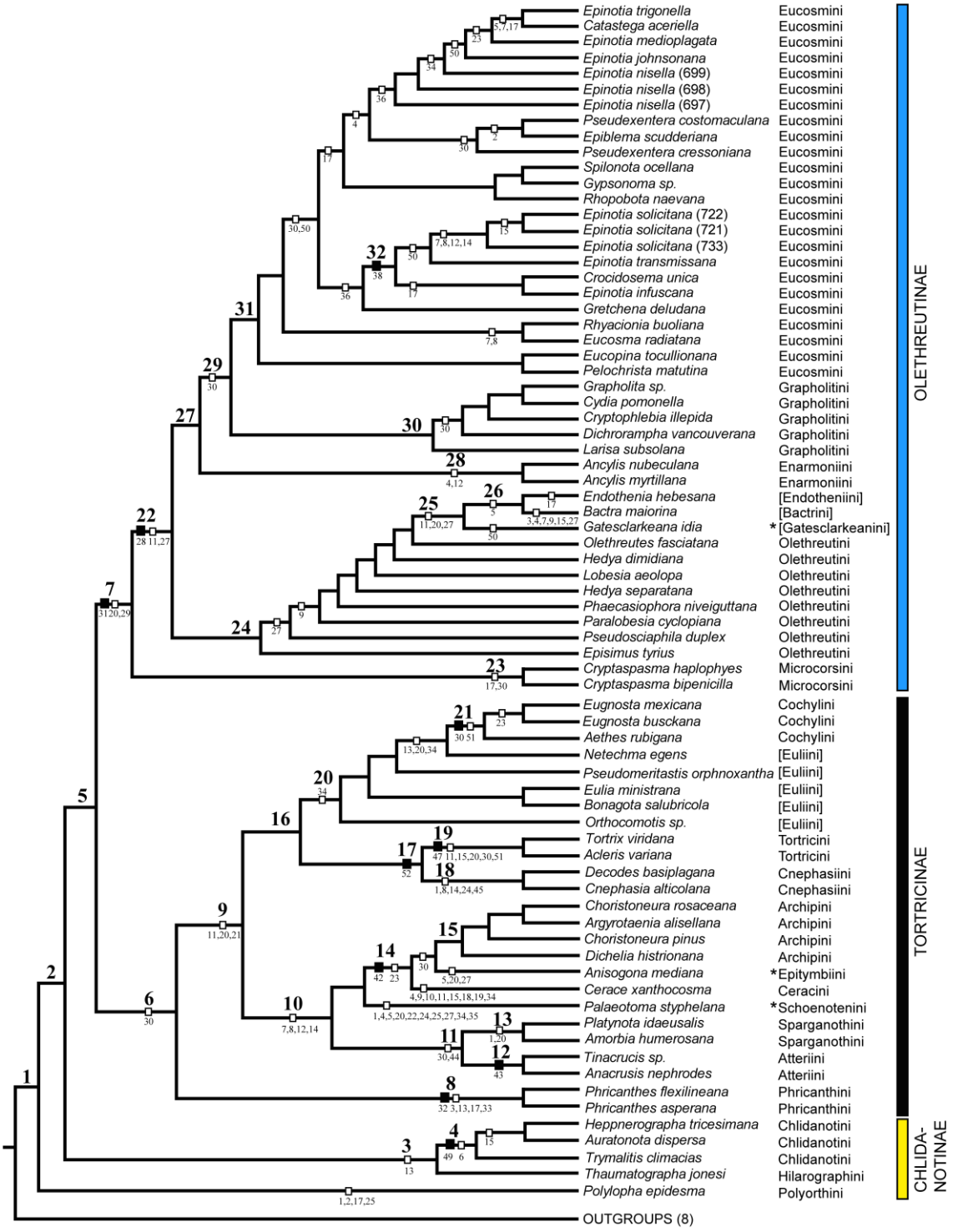

Figure 3.3 Tree topology based off Regier *et al.* (2012) phylogeny (length=411, CI=0.2019, RI=0.5874) showing morphological character distribution.

Notes: Filled blocks indicate a synapomorphies, open blocks indicate homoplasious apomorphies. Clades are identified by numbers 1-32.

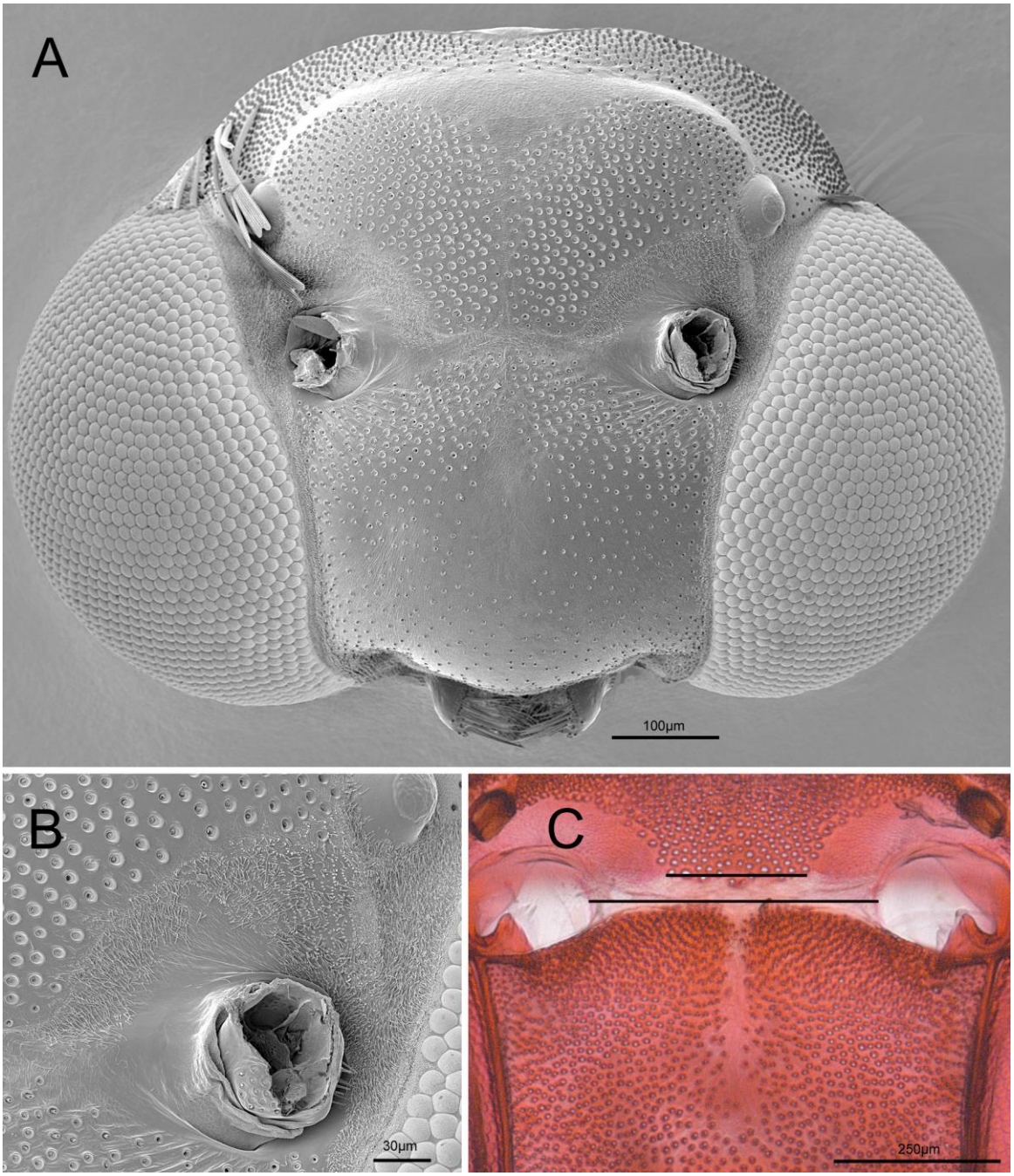

Figure 3.4 Characters of the head

Notes: A, head of *Bactra maiorina*. B, antennal socket of *B. maiorina* surrounded by microtrichia C, head of *Anacrusis nephrodes* with bars to indicate measurements for character 34.

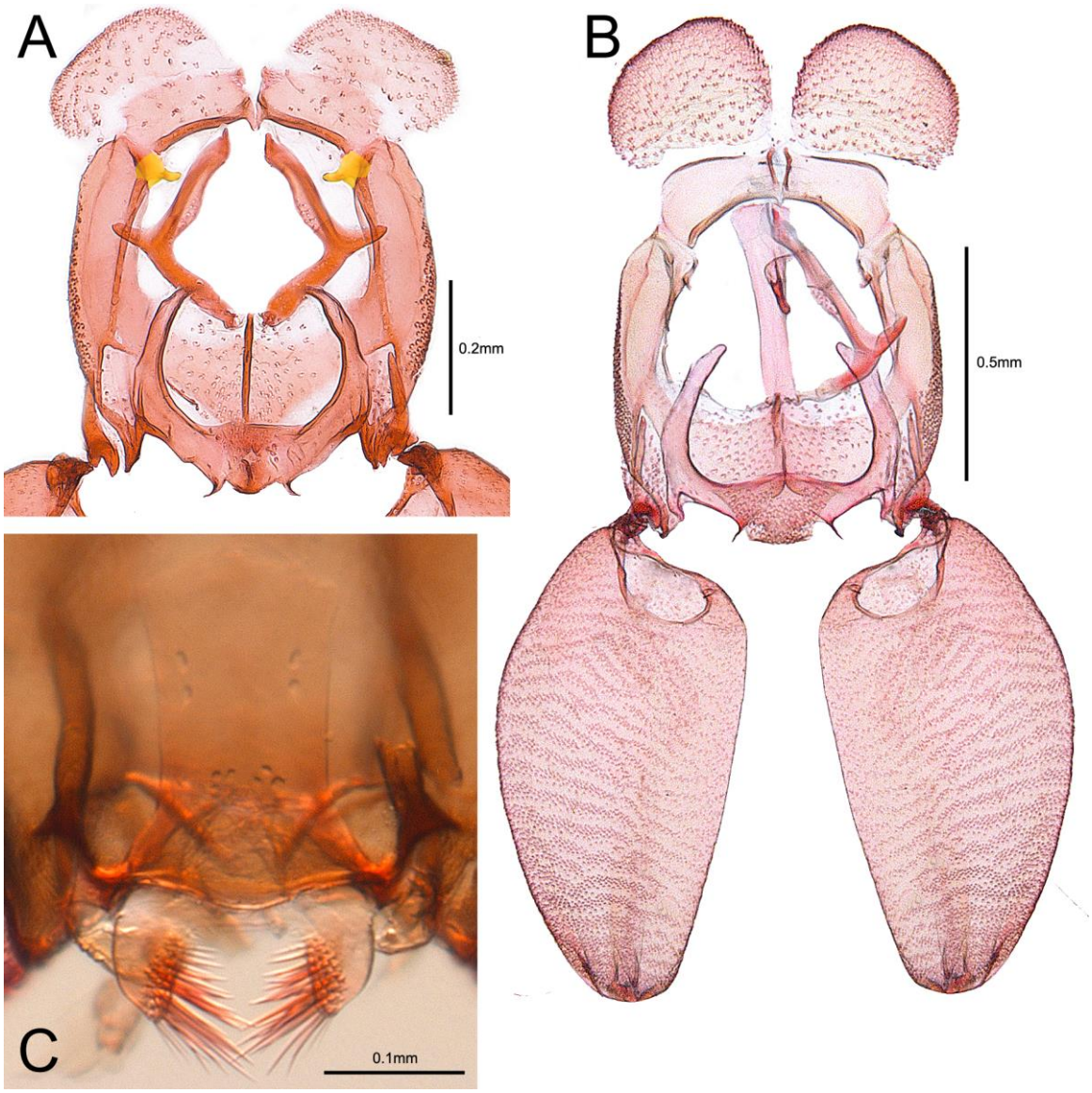

Figure 3.5 Characters of the head and prothorax

Notes: A, posterior view of prothorax in *Bactra maiorina* with apophyses highlighted yellow. B, posterior view of prothorax in *Phricanthes flexilineana* with posterior pointing apophyses. C, dorsal view of sitophore in *B. maiorina*.

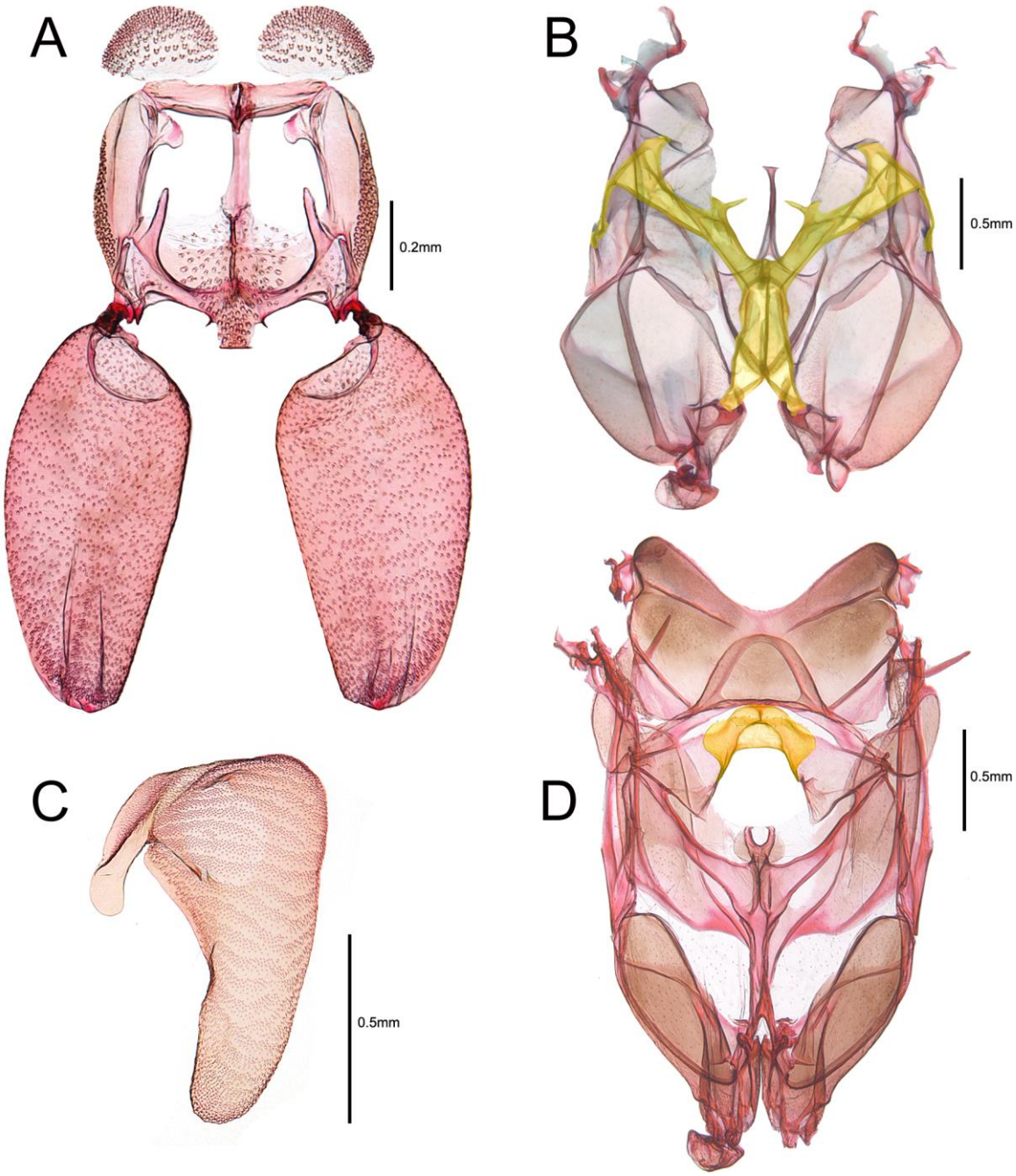

Figure 3.6 Characters of the pro-, meso-, and metathorax

Notes: A, posterior view of prothorax in *Gatesclarkeana idia* with scale sockets arranged in rows on the procoxa. B, posterior view of mesothorax in *Hedya dimidiana* with mesofurca highlighted yellow. C, tegulae of *Phricanthes flexilineana* with scale sockets arranged in rows. D, posterior view of metathorax in *Eugnosta busckana* with euphragma, with lateral flanges, highlighted yellow.

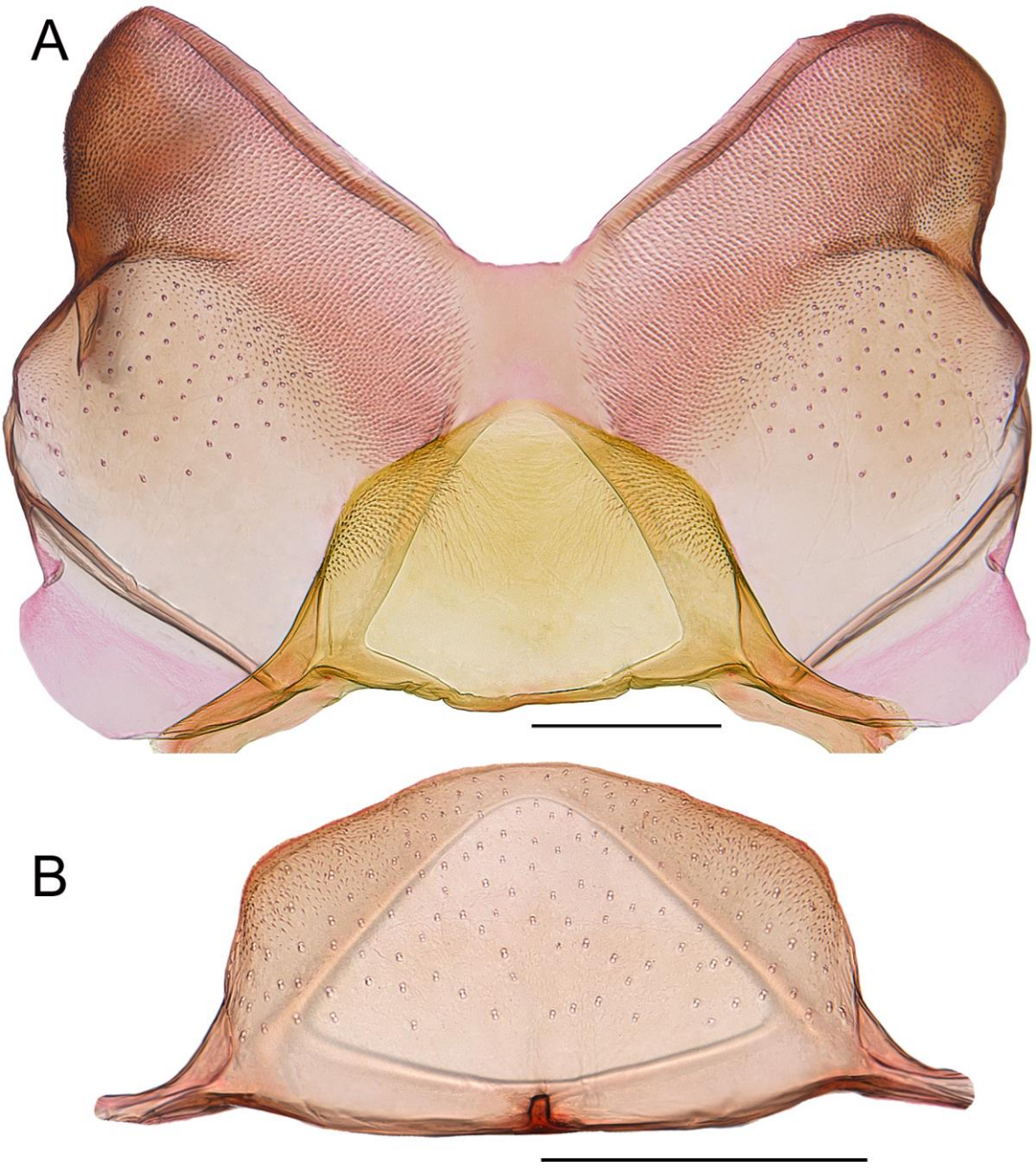

Figure 3.7 Characters of the metathorax

Notes: A, dorsal view of metascutellum+metascutum in *Heppnerographa tricesimana.* B, dorsal view of metascutellum in *Phricanthes flexilineana.* Scale bar = 0.2mm

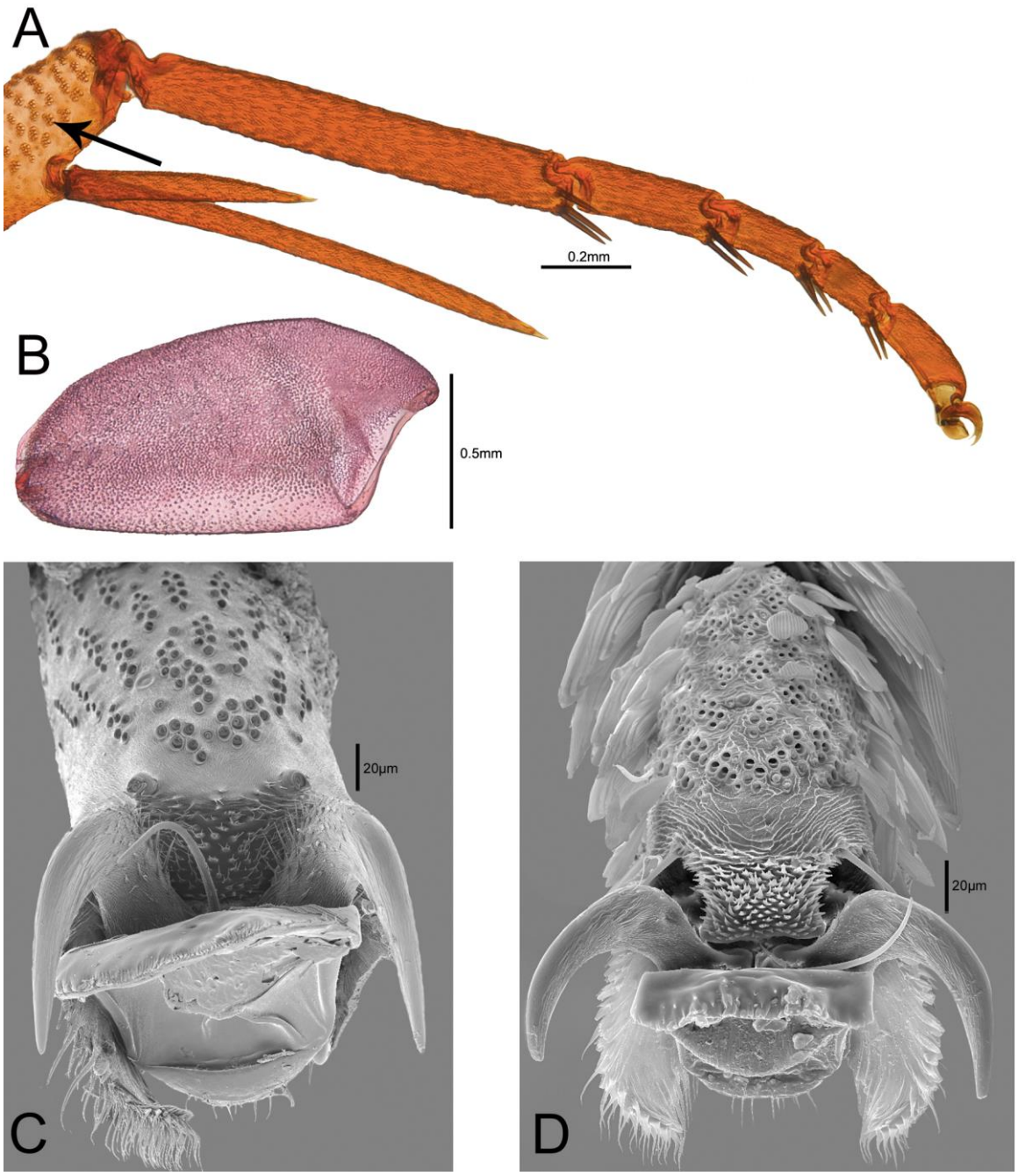

Figure 3.8 Characters of the legs

Notes: A, lateral view of metaleg in *Cydia pomonella* showing three spiniform setae per tarsal segment, and round groups of scale sockets on the tibia indicated by an arrow. B, anterior view of procoxa in *Cryptaspasma bipenicilla.* C, apicodorsal view of proleg claw in *Orthocomotis sp.* with elongate groups of scale sockets on the tarsomere. D, apicodorsal view of the proleg claw in *C. bipenicilla* with round groups of scale sockets on the tarsomere.

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APPENDIX A

ADDITIONAL FIGURES DETAILING CHARACTER STATE DISTRIBUTION FOR ALL TAXA

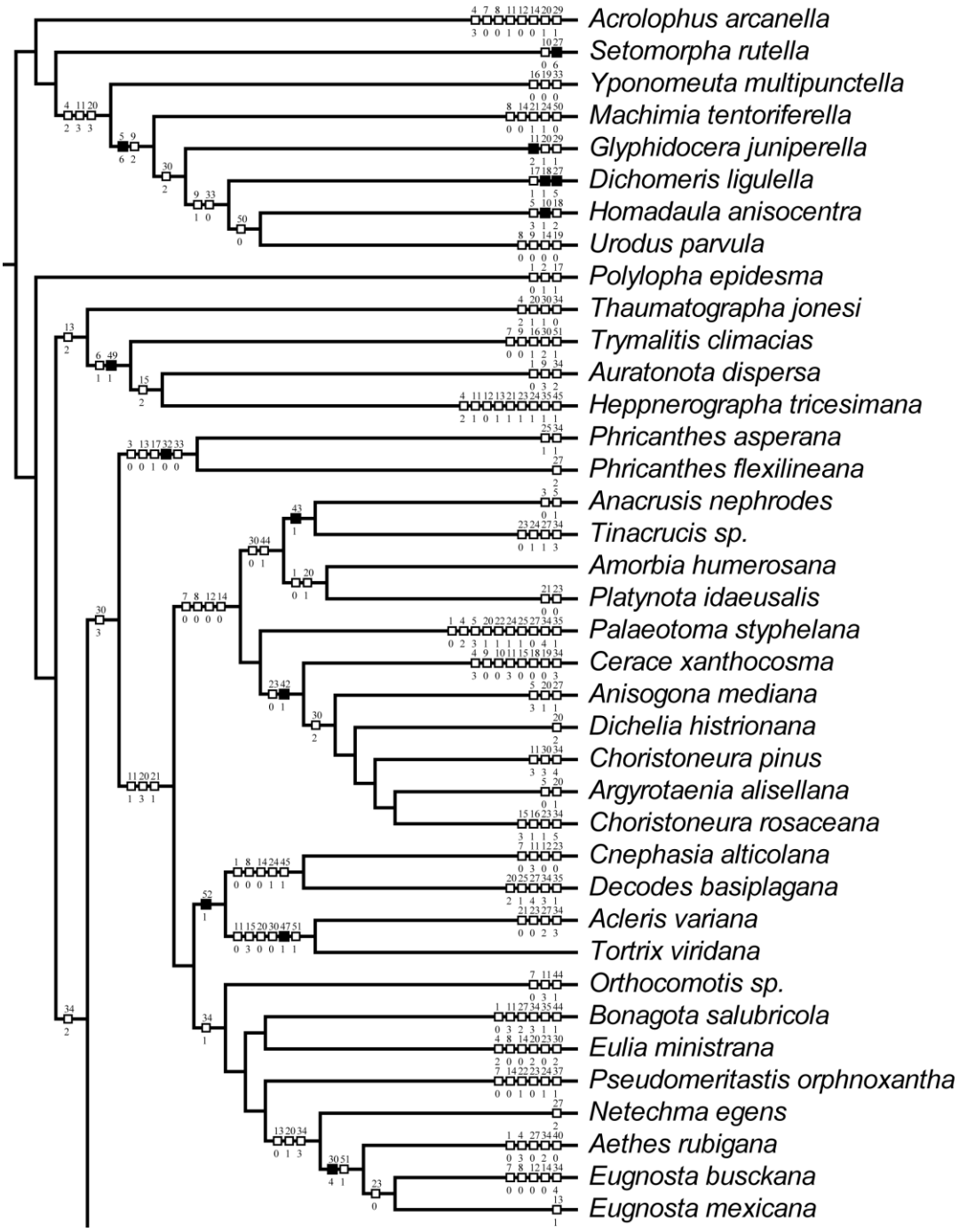

Figure A.1 Tree topology based off Regier *et al.* (2012) phylogeny (length=411, CI=0.2019, RI=0.5874) showing morphological character distribution for all branches within the outgroups, Chlidanotinae, and Tortricinae.

Notes: Filled blocks indicate a synapomorphies, open blocks indicate homoplasious apomorphies.

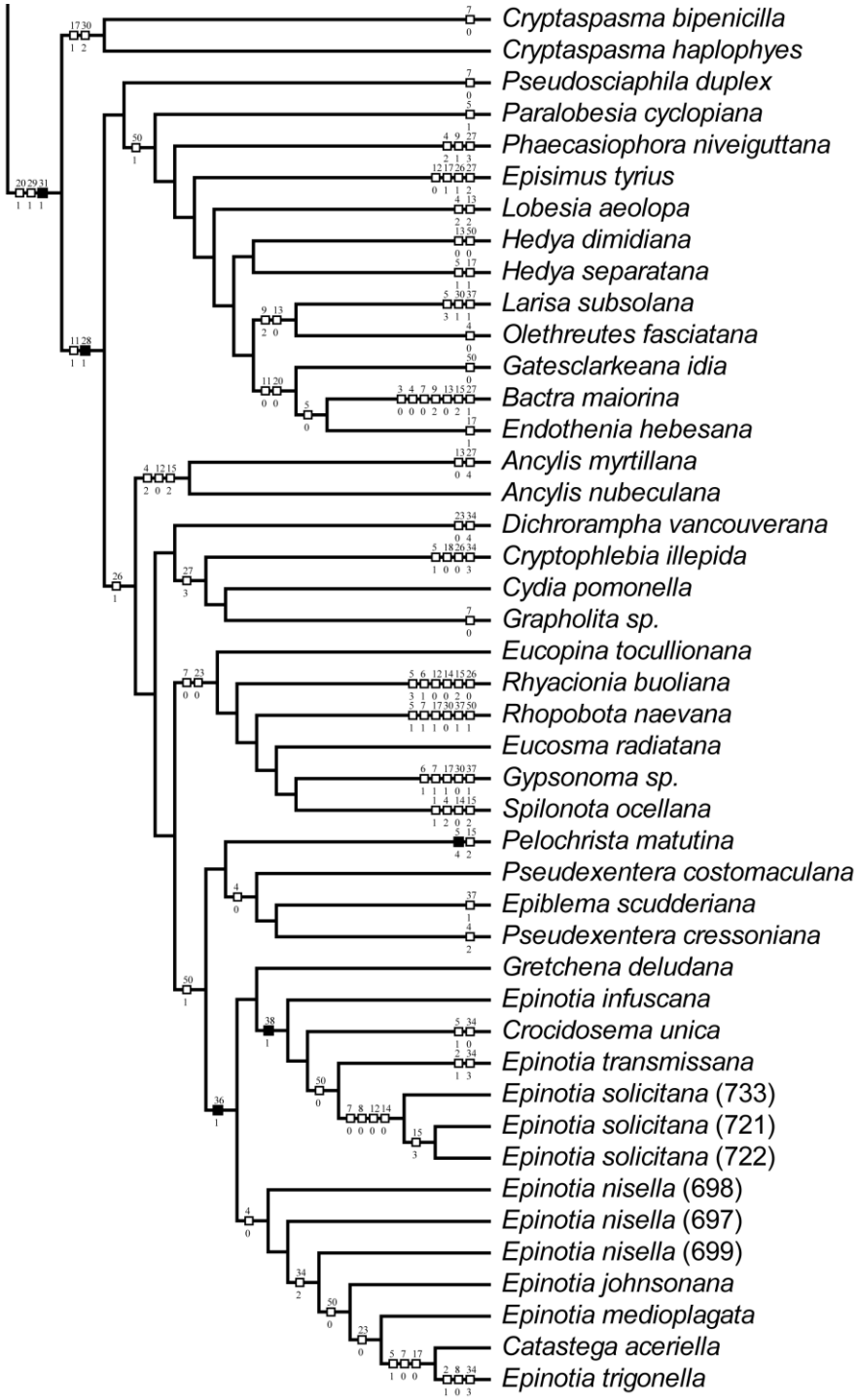

Figure 3.9 Tree topology based off Regier *et al.* (2012) phylogeny (length=411, CI=0.2019, RI=0.5874) showing morphological character distribution for all branches within Olethreutinae.

Notes: Filled blocks indicate a synapomorphies, open blocks indicate homoplasious apomorphies.