

INVESTIGATING THE SUBSPECIFIC DESIGNATION OF *EUSCHISTUS SERVUS* (SAY)
(HEMIPTERA: HETEROPTERA: PENTATOMIDAE) USING A COMBINED LANDMARK-
BASED GEOMETRIC MORPHOMETRICS APPROACH

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ABSTRACT

Euschistus servus is currently subdivided into two subspecies: *E. s. servus* and *E. s. euschistoides*. An intergrade population inhabits the central U.S. This study sought to reevaluate the established subspecific designations and determine whether *E. servus* subspecies and intergrade populations express distinct taxonomically important morphological characteristics throughout the U.S. We quantified landmark-based geometric morphometric data in conjunction with traditional morphometrics from specimens collected along a wide geographic area and used a principal component analysis to determine if there is a significant difference within the subspecies' morphology, assessing shape between morphogroups failed to produce clusters commensurate to the *E. s. servus* and *E. s. euschistoides* forms. Further analysis suggested that there is linear relationship between the taxonomic characters and latitude which may be indicative of clinal variation. This data indicates *E. servus* should be recognized as polymorphic as opposed to polytypic, disputing the subspecific designation of the *E. servus* subspecies complex.

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DEDICATION

I would like to dedicate this to my mentors, my colleagues, my parents, my partner, and my puppy.

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LIST OF DEFINITIONS

aedeagus.....	reproductive organ of male arthropods through which they secrete sperm from the testes during copulation with a female.
allometry.....	the growth of body parts at different rates, resulting in a change of body proportions.
antennate.....	act of touching the antennae to another insect.
arbitrary.....	choice made without any specific criterion or restraint.
archetypal.....	very typical of a certain kind of organism.
chorion.....	outer shell of an insect's egg. May consist of several layers including a waxy layer to prevent water loss.
cline.....	a gradation in one or more characteristics within a species or other taxon, especially between different populations.
copulation.....	sexual intercourse.
courtship.....	behavior by which different species select their partners for reproduction.
cryptic species.....	group of species that contain individuals that are morphologically identical to each other but belong to different species under the biological species concept.
diapause.....	delay in development in response to regularly and recurring periods of adverse environmental conditions.
dimorphism.....	existence of two different forms (as of color or size) of a species especially in the same population.
genotype.....	organism's complete set of genetic material; may refer to an organism's single gene or set of genes.
fuscous.....	dark and somber in color; brownish-gray or dusky color.

geometric morphometrics	approach that studies shape using Cartesian landmark and semi-landmark coordinates that can capture morphologically distinct shape variables.
hemelytra.....	modified hardened forewing; basal section thickened and apex membranous.
hemimetabolous	incomplete metamorphosis.
incipient species	particular species that are about to become genetically isolated from the rest of the species, perhaps due a geographical barrier, though at this time can still reproduce with other groups of the species before their gene pools become too distinct..
intergrade	an intermediate form.
metabolite.....	in biochemistry, a metabolite is an intermediate or end product of metabolism.
morphometrics	measurement and analysis of form.
multivariate	involving two or more variable quantities.
operculum	circular lid that constitutes the upper portion of the egg and functions as an exit for the emerging nymph.
phenology.....	study of periodic events in biological life cycles and how these are influenced by seasonal and interannual variations in climate, as well as habitat factors.
phenotype.....	set of observable characteristics of an individual resulting from the interaction of its genotype with the environment.
photoperiod	the period each day during which an organism receives illumination (day length).
phylogenetic	relating to the evolutionary development and diversification of a species or group of organisms, or of a particular feature of an organism.
phytophagous	plant feeding.
polyphagous	(of an herbivorous insect) Having many host plants.

synonym.....scientific name that applies to a taxon that (now) goes by a different scientific name.

systematicsbranch of biology that deals with classification and nomenclature; taxonomy.

taxon.....group of one or more populations of an organism or organisms seen by taxonomists to form a unit.

taxonomy.....scientific study of naming, defining (circumscribing) and classifying groups of biological organisms based on shared characteristics.

trinomenin zoological nomenclature refers to the name of a subspecies.

vagilitydegree to which an organism or taxon can or does move or spread within an environment.

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Humans are hard wired to categorize everything. It is a key cognitive ability that allows us to organize our thoughts, perception, action, and speech, which effectively shapes the way we understand the world. We utilize categories whether we are simply writing or employing something more complex (*e.g.*, any of the species' concepts, Table 1.1). Whether we ascribe to Aristotle's classic theory of classification, or to Rosch's 1973 prototype theory, we find that most categorization happens automatically and unconsciously, it is only in intricate situations where we become aware of it at all (Lakoff 1987). With categorization at the basis of thought and inherently tied to the human psyche, and one of the key necessities of science, especially systematics, there's no wonder we find biodiversity captivating. As categories may be formed unconsciously through associations and imagination (Lakoff 1987) it is also unsurprising that there is disagreement on the definitions among some of the finer categories of taxonomy. As necessity is the mother of invention, it is also unsurprising that biologists and systematists have created numerous methods to delineate subspecies aside from utilizing morphological significant characteristics ranging from classical morphometrics, geometric morphometrics, genomics, phylogenetics, and various statistical methods. The subspecies concept has been in use for nearly two centuries and continues to be a very controversial topic in systematics.

Since Hermann Schlegel's first application of a trinomen to designate geographic subdivisions of species in 1844 (Sibley 1954) the subspecies has been described and implemented in numerous ways (Table 1.1). There has been an academic battle over the definition and application of the widely used concepts of a subspecies, particularly within the Class Insecta (Remsen 2010). Simpson (1961) contends that all the arguments boil down to two

questions “Is there any objective basis for subspecies?” and, “Is this recognition of a formal taxonomic subcategory useful to taxonomists?” His answer: “Yes and no.” Simpson suggests that there is sometimes an objective basis for subspecies and sometimes there is not. He also suggests that formal recognition of subspecies as a lower taxonomic rank may be as helpful as it is unhelpful. Several authors have written outstanding comprehensive literature reviews (Keita 1993; Winker 2010; Vinarski 2015a, b), on the history of subspecific study, (*i.e.*, microsystematics), and answered the big questions of this nearly two century old controversy, namely, “What are subspecies?,” “How do we diagnose a subspecies?,” “What does subspecific variation mean?,” and “Is the simple scheme of species and its subspecies sufficient?” While this research attempts to model methods that may help to standardize the application of the subspecies concept using landmark based geometric morphometrics, this literature review covers several topics, including: 1) reviewing the taxonomic classification and life history of the brown stink bug, *Euschistus servus* (Say) (Hemiptera: Pentatomidae); 2) a brief history of the subspecies concept, its application, issues, their resolutions, and the revival in arguments over the past decade; and 3) the methodology used to investigate the subspecific designation within *E. servus*.

Table 1.1. Subspecies definition from the literature. (modified from Remsen 2010).

Definition	Source
Geographically defined aggregates of local populations which differ taxonomically from other such subdivision of species	Mayr <i>et al.</i> 1953, Mayr 1963
A set of populations of a species that share one of more distinctive features and occupy a different geographic area from other subspecies	Futuyma 1979
An aggregate of local populations of a species inhabiting geographic subdivision of the range of the species and differing taxonomically [differing by sufficient diagnostic characters] from other populations of the species	Mayr and Ashlock 1991
A taxonomic division of a species often distinguished by special phenotypic characters and by its origin or localization in a given geographic region	Strickberger 2000
A recognizably distinct population, or group of populations, that occupies a different geographic area from other populations of the same species; populations of a species that are distinguishable by one or more characteristics and are given subspecific names	Futuyma 2005
Proposes to redefine the concept; Subspecies as a type of species; not a lower taxonomic rank. Subspecies are members of the same category as species that are nested within other members of that category. Trinomials to be used as a representational device to indicate the nesting of incompletely separated lineages with a more inclusive lineage	de Queiroz 2020

1.2. Taxonomy, Life History, and Biology of *Euschistus servus*



Figure 1.1. Image of *E. s. servus* (© 2017 Mike Quinn)

1.2.1. Taxonomic Info

Order: Hemiptera

Suborder: Heteroptera

Infraorder: Pentatomomorpha

Superfamily: Pentatomoidea

Family: Pentatomidae

Subfamily: Pentatominae

Tribe: Carpocorini

Genus: *Euschistus* Dallas

Species: *servus* Say

Subspecies: *E. s. servus* and *E. s. euschistoides*

1.2.1.1. Higher Classification

The Heteroptera, or the true bugs, were initially founded by Latreille (1810) as a suborder of Hemiptera. This suborder is the most speciose hemimetabolous (incomplete metamorphosis) insect taxa. There are 91 families consisting of over 45,000 heteropteran species (Henry 2017) that occur on all land masses, except Antarctica (Schuh and Weirauch 2020). Heteroptera is generally considered to be monophyletic group defined by several characteristics: 1) hemelytra (wings partially sclerotized basally and partially membranous distally) usually positioned flat over the mesothorax, metathorax, and the abdomen; 2) a rostrum, or beak, attached anteriorly on the head; 3) antennae consisting of four or five segments; 4) a well-developed scutellum; and 5) a usually well-developed pair of metathoracic scent glands in adults, and dorsal abdominal scent glands in nymphs (Schuh and Weirauch 2020). The Heteroptera usually is divided into seven infraorders, although some have argued for an eighth infraorder (*i.e.*, Aradimorpha) (Sweet

2006). These infraorders are further divided into at 24 superfamilies (Henry 2017). *Euschistus servus* falls within the infraorder Pentatomomorpha, and the superfamily Pentatomoidea (Fig. 1.1).

1.2.1.2. Infraorder and Superfamily

The Pentatomomorpha was first established by Leston *et al.* (1954); it traditionally includes six superfamilies: Aradoidea (sometimes considered its own infraorder), Idiostoloidea, Pentatomoidea, Lygaeoidea, Pyrrhocoroidea, and Coreoidea. *Euschistus servus* falls within the Pentatomoidea. This superfamily is comprised of 16 extant families and two fossil families; it also contains 1410 genera and 8042 species globally (Rider *et al.* 2018). Within North America, the pentatomoids are represented by five native families and one introduced family: the native Acanthosomatidae, Cydnidae, Pentatomidae, Scutelleridae, and Thyreocoridae (Henry 2017) and the inadvertently introduced Plataspidae (Eger *et al.* 2010). The Pentatomoidea and Aradoidea are consistently accepted as monophyletic groups with the phylogenetic relationships of the other superfamilies uncertain (Xie *et al.* 2005).

1.2.1.3. Family

The Pentatomidae is the most speciose family within the five Pentatomoidea superfamilies in North America containing 223 phytophagous and predaceous species within five subfamilies (*i.e.*, Asopinae, Discocephalinae, Edessinae, Pentatominae and Podopinae) within 68 genera (Rider and Swanson 2021). Globally, the Pentatomidae contains 4949 species in 940 genera (Rider *et al.* 2018). *Euschistus servus* belongs to the subfamily Pentatominae, the most speciose subfamily.

1.2.1.4. Genus

Euschistus Dallas, currently placed in the tribe Carpocorini, occurs exclusively in the New World and contains several economically important species (Munyaneza and McPherson 1994, Bianchi *et al.* 2017). The Neotropical species of *Euschistus* have been revised relatively recently in two parts: for Middle America (Rolston 1974), and South America (Rolston 1985); although McPherson (1982) provided a key to those species occurring in northeastern North America, there is no modern work concerning all North American species. Although they are easily recognized as a stink bug, the delineation among *Euschistus* species can be quite difficult. The 19 species of *Euschistus* distributed throughout North America (Rider and Swanson 2021) are characterized as being medium-sized, brown, reddish brown, yellowish brown, or grayish in color with a strong peltate (shield-like) shape (Blatchley 1926, McPherson 1982). The ventral surfaces are often similarly but lightly colored, occasionally speckled with red (Rolston 1974). Rolston (1974) noted that the relative lengths of the tylus and juga were usually fairly uniform in most species of *Euschistus*. In the case of *Euschistus servus*, however, these relative lengths may vary. *Euschistus servus* is one of the most ubiquitous phytophagous members of the Pentatomidae in North America.

1.2.1.5. Species

Euschistus servus is distributed throughout North America and is currently subdivided into two subspecies *Euschistus servus servus* (Say 1831) in the southern U.S., historically found south of 40° latitudes, and *Euschistus servus euschistoides* (Vollenhoven 1868) in the northern U.S., historically found north of 40° latitudes, as well as an intergrade population stretching from Maryland to Kansas (Fig. 1.2). The subspecies range from 11.0 to 15.0 mm in length (*i.e.*, from the apex of the genital segment to the tip of tylus) (Fig. 1.3), and are distinguished primarily by

differences in the color of the antennal segments, exposure of the connexiva, and the differences in the relative lengths of the juga and tylus (Blatchley 1926, McPherson 1982). There may also be some subtle differences in the shape in the humeral angles of the pronotum and apparent variation in abdominal width, but this might be confounded with the differences in connexival exposure (Blatchley 1926).

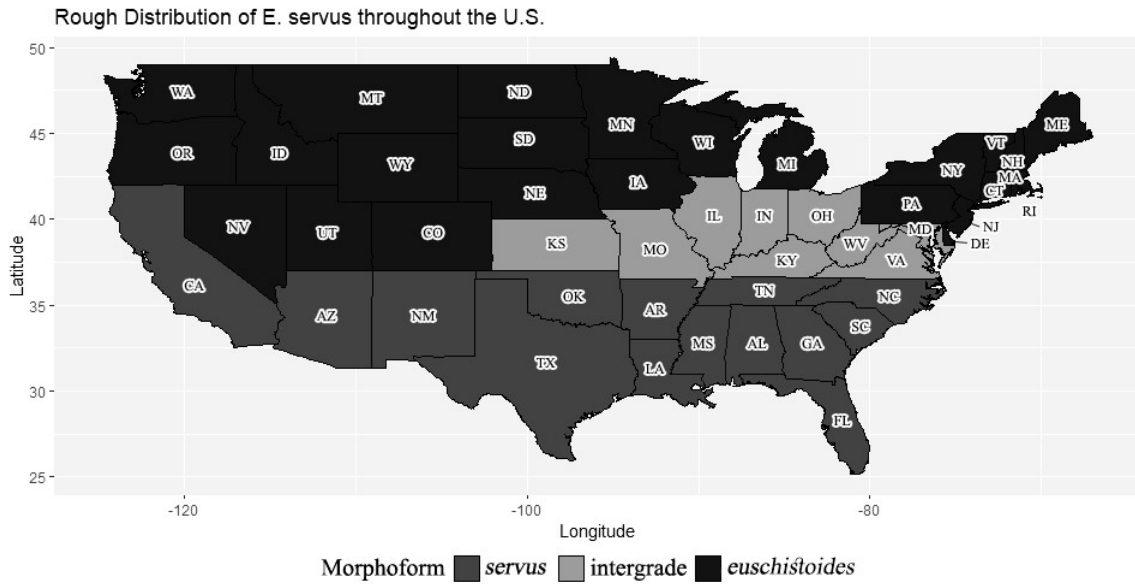


Figure 1.2. Distribution of *E. servus* throughout the United States (modified from McPherson 1982).

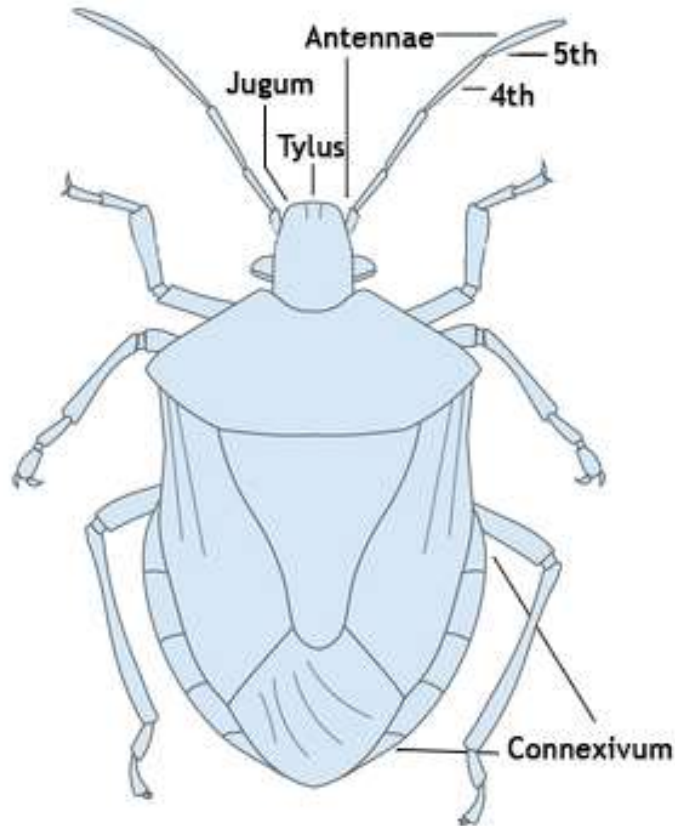


Figure 1.3. Dorsal view of a stink bug depicting key morphological features used to delineate subspecies of *E. servus* (adapted from Kobayashi 1967, image used with permission from Oregon Department of Agriculture).

Euschistus s. euschistoides specimens generally have juga that are longer than the tylus, antennal segments 4 and 5 are usually dark, and the connexiva is usually completely covered by the hemelytra. Whereas, *Euschistus s. servus* specimens generally have juga that are subequal in length to the tylus, antennal segments 4 and 5 are entirely yellowish or reddish brown, and the connexiva is broadly exposed. The intergrade population is recognized as having characteristics intermediate between those listed above (*e.g.*, partially exposed connexiva) (Fig. 1.4) (Sailer 1954, McPherson 1982).

Owing to the variability in coloration, and the form of several morphological characters both subspecies have been described multiple times (Table 1.2). For example, Stål (1872) described *E. impictiventris* (now considered to be a synonym of *E. s. servus*), based on

differences in coloration, and in the form of the humeral angles. Rolston (1974) noted that the general coloration of the dorsum apparently follows a cline that conforms to Gloger's Rule of variation (*i.e.*, variation in animal coloration relates to broad-scale climatic gradients; organisms tend to be darker in humid and warm environments compared to colder and drier areas) (Delhey 2017). In addition, the acute humeral angles described by Stål are not confined to specimens from the southwestern range, our data demonstrates that these specimens have been found elsewhere in its range.

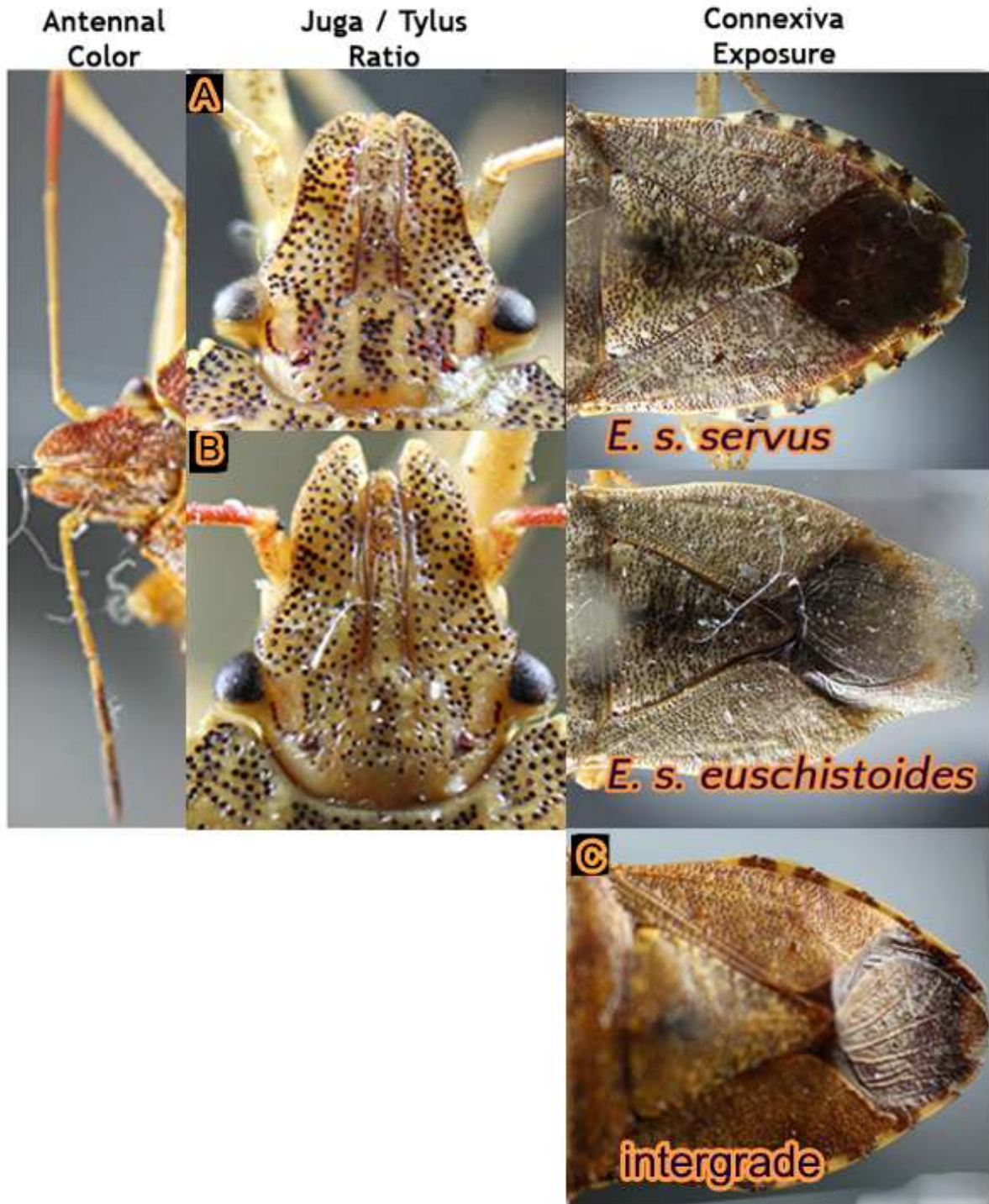


Figure 1.4. Morphological differences between the A) *E. s. servus*, B) *E. s. euschistoides* C) intergrade forms.

Table 1.2. Noted synonyms found in the literature for each subspecies.

Subspecies	Synonym	Author	Year
<i>Euschistus servus euschistoides</i>	<i>Diceraeus euschistoides</i>	Vollenhoven	1868
	<i>Euschistus fissilis</i>	Uhler	1871
	<i>Euschistus jugalis</i>	Provancher	1889
<i>Euschistus servus servus</i>	<i>Pentatoma serva</i>	Say	1831
	<i>Pentatoma harrisii</i>	Westwood	1837
	<i>Pentatoma spilota</i>	Westwood	1837
	<i>Euschistus impictiventris</i>	Stål	1872
	<i>Euschistus subimpunctatus</i>	McAtee	1919

1.2.2. Life History and Biology of *Euschistus servus*

The *E. servus* subspecies complex has a contiguous continental range and is commonly found throughout North America. *Euschistus. servus* overwinters as an adult under crop residues, in forest detritus, and various weeds (Rolston and Kendrick 1961, McPherson 1982), showing a preference to overwinter in open fields as opposed to woodlands or the field-woodland edge (McPherson and McPherson 2000). The adults undergo facultative diapause induced primarily by photoperiod (Borges 2001, Saulich and Musolin 2011), which is associated with a change of adult ventral color from green to reddish brown. Mating and egg deposition resume in the spring shortly after adults emerge from overwintering sites, also cued by a change in photoperiod (McPherson 1982, Borges 2001). Fecundity, fertility, and copulatory behavior has been studied in previous studies (*e.g.*, see Youther and McPherson 1975, Drickamer and McPherson 1992).

While there may be a general mating pattern in stink bugs, inter and intraspecific variations exist in courtship behavioral patterns (*e.g.*, *E. servus* has not been recorded performing the winged behavior that is found in some other stink bug species such as *Dichelops melacanthus* [Dallas]) (Drickamer and McPherson 1992). *Euschistus servus* exhibits a specific set of copulatory behavioral patterns, characteristics, and cues (Table 1.3) that distinguish the species. *Euschistus servus* produces vibrational signals via repeated muscle contractions that cause the

abdominal plates to vibrate. These vibrations are transmitted over the thorax and legs through the substrate for medium-range location (calling) and short range (courtship) (Čokl 2008, Lampson *et al.* 2010). Each song is specific to a stage in the courtship process. This species has several recorded songs, two songs distinct for males and four songs distinct for females differing in mean dominant frequency, duration, and repetition time (Lampson *et al.* 2010). Courtship is initiated with the male antennating the female's antennae, head and pronotum, followed by antennation of her thorax and abdomen. If the female is receptive, she will raise her abdomen for aedeagal (penile) insertion. Often copulation ends in an end-to-end position with the female dragging the male along. *Euschistus servus* exhibits a relatively longer duration of copulation lock which suggests mate guarding (preventing their mates from receiving genetic material from rivals via prolonged copulation after insemination) (Drickamer and McPherson 1992, Alcock 1994). If the female is unreceptive, she will not raise her abdomen and will generally kick the male away from her (Youter and McPherson 1975, McPherson 1982, Drickamer and McPherson 1992, McPherson and McPherson 2000).

Table 1.3. Summary of primary mating sequences of six species of stink bugs (Drickamer and McPherson 1992).

Species	No. Sequences	Direction	Latency to lock	Duration of lock
<i>E. ictericus</i> (L.)	2+	head to rear	long	long
<i>E. politus</i> Uhler	1	head to rear	short	short
<i>E. servus</i> (Say)	1	head to rear	long	intermediate / long
<i>E. tristigma</i> (Say)	2+	both	long	intermediate / long
<i>E. variolarius</i> (P. de B.)	1	head to rear	short	intermediate
<i>Thyanta custator accerra</i> McAtee	1	rear to head	short	long

After successful copulation, *E. servus* will generally oviposit a clutch of eggs on the underneath surface of the host plant's leaves. Like other pentatomids, the eggs of *E. servus* are

oviposited in clusters in relatively consistent patterns. They can be recognized by their cylindrical or spherical barrel-shape, and a distinct coloration and morphology of the chorional surface (Esselbaugh 1946; Matesco *et al.* 2009, 2014). For example, the eggs may be characterized by their cream or white color, a spinose chorion with triangular and quadrangular reticulations and an operculum (*i.e.*, a circular lid that constitutes the dorsal portion of the egg and functions as an exit for the emerging nymph) present (Esselbaugh 1946, Munyaneza and McPherson 1994, Bundy and McPherson 2000a). The nymphal stages generally consist of five instars, taking about 33 days to develop (Fig. 1.5) (Rolston and Kendrick 1961). The eggs and adults are often attacked by several families of parasitoids (*e.g.*, sarcophagid flies, tachinid flies, encyrtid wasps, and scelionid wasps) and predated upon by asilid flies, nyssonid wasps, and an asopine pentatomid (McPherson 1982). *Podisus maculiventris* (Say) (spined soldier bug) is a generalist predator having roughly the same distribution as *E. servus*. It has been recorded as feeding on over 90 insect species spanning eight orders, including nymphs and adults of *E. servus* and other pentatomids (McPherson 1982, De Clercq 2000, Tillman and Mullinix 2004, Koch *et al.* 2017).

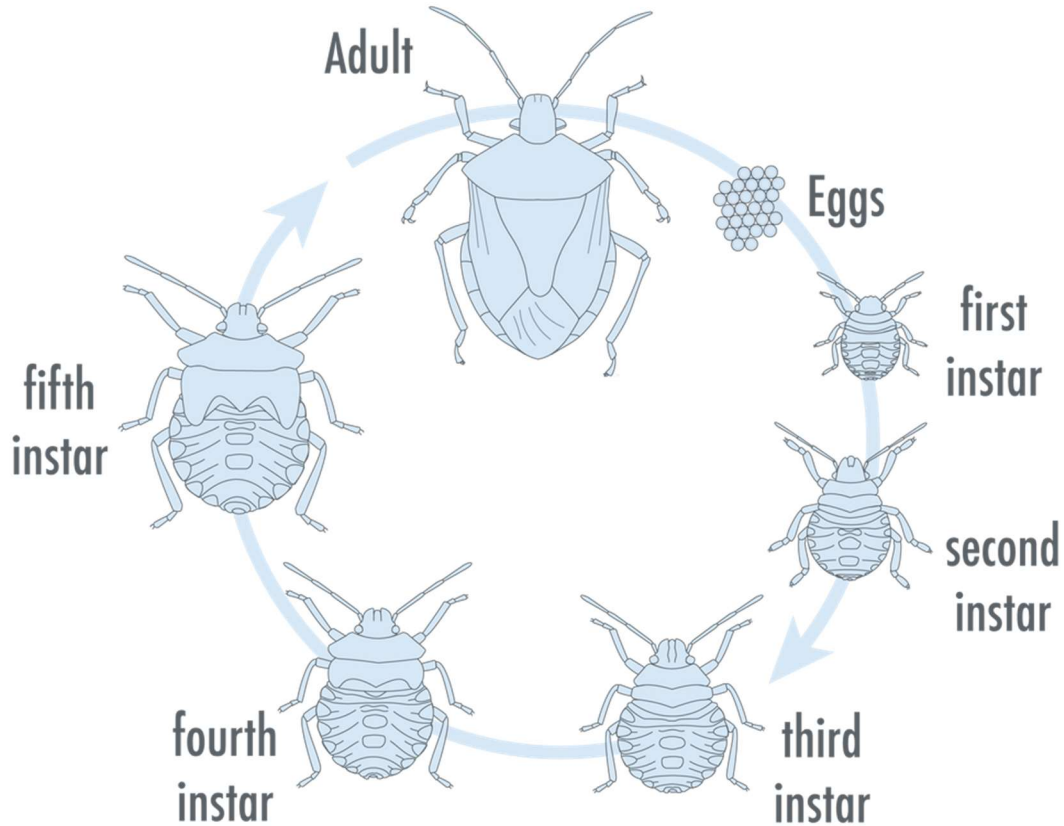


Figure 1.5. Pentatomidae life cycle (Adapted from illustrations in Kobayashi 1967 by Oregon Department of Agriculture).

Like other pentatomid species, *E. servus* possesses highly developed abdominal and metathoracic scent glands (MTG) where they sequester secondary plant metabolites which are used primarily as defensive chemicals to ward off invertebrate and vertebrate predators. They may also be used in intraspecific communication (*i.e.*, as pheromones), and as protection against microorganisms (Staddon 1979, Aldrich 1988). While the form of the MTGs exhibit a general pattern throughout the Heteroptera, there is inter- and intraspecific morphological and chemical variation (Staddon 1979), including active and diapausing morphs within a species (Hassani *et al.* 2010). The glandular origins for the pheromones of *Euschistus* spp. is relatively unstudied. The chemical composition of their pheromone, however, has been studied; the primary component of male-specific volatiles of all North American *Euschistus* spp. that have been

studied (except for *E. obscurus* [Palisot de Beauvois]), has been identified as methyl (2E, 4Z) – decadienoate ($C_{11}H_{18}O_2$) (Fig 1.6) (Aldrich *et al.* 1991).

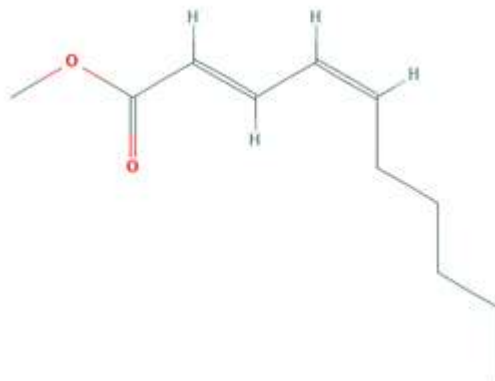


Figure 1.6. 2D Chemical structure depiction of methyl (2E,4Z)-2,4-decadienoate which is also used as a food flavor additive (PubChem).

1.3. Economic Importance

Stink bugs (Hemiptera: Pentatomidae) were recorded as crop pests as early as 1854 (Morril 1910). *Euschistus servus* are voracious polyphages and feed on many different host plants throughout North America, occurring on numerous wild and domesticated plants some of which are important (Greene *et al.* 2001), including economically significant crops (*e.g.*, cabbage, corn, okra, peas, pecan, snap peas, soybean, tomatoes) and weeds (*e.g.*, Canada thistle, reed canary grass, yellow thistle, white clover) (Table A1). *Euschistus servus* has also been reported as occasionally exhibiting predaceous behavior with records of it feeding on *Alabama argillacea* (Hübner) (Lepidoptera: Erebidae), (Riley 1885), *Pristiphora geniculata* (Hartig) (Hymenoptera: Tenthredinidae) (Beaulne 1939, Forbes and Daviault 1964), and *Pieris rapae* (Linnaeus) (Lepidoptera: Pieridae) (Culliney 1985). Stink bugs feed by piercing food items with the mandibular and maxillary stylets in their beak, typically resulting in spot like blemishes, decreased nutrition content, and lower crop yield (Daugherty 1967). Additionally, the deposition of their defensive secretions on the plant surface can further devalue crops (Rolston and

Kendrick 1961, McPherson 1982). Stink bugs may also vector certain plant diseases. Examples include yeast-spot disease (*Eremothecium coryli* [Peglion]) (Daugherty 1967 as *Nematospora coryli*) and cotton boll rotting bacteria (*Pantoea agglomerans* [Beijerinck]) (Medrano *et al.* 2016). Introducing genetically modified cultivars of cotton expressing *Bacillus thuringiensis* Berliner (Bt) toxins to help control damage by the tobacco budworm (*Chloridea virescens* [Fabricius]) and the cotton bollworm (*Helicoverpa zea* [Boddie]) alongside attempts to eradicate the boll weevil (*Anthonomus grandis* Boheman) reduced the need and application of insecticides targeting pests in the southeastern United States (Greene *et al.* 2001), which then led to an increase in abundance of *E. servus* and other pentatomids in the region, especially in cotton (Bundy and McPherson 2000b, Ridge *et al.* 2000). This increase in abundance correlated with an increase in crop damage, with \$31 million in losses recorded on cotton in 2008 and \$60 million in losses on soybean annually (Pilkay *et al.* 2015). As a result, *E. servus* succeeded in becoming one of the most important stink bug pests alongside *Nezara viridula* (Linnaeus) (southern green stink bug) and *Chinavia hilare* (Say) (green stink bug) (Bundy and McPherson 2000b).

Their varied feeding preferences allow them to move between cultivated and uncultivated hosts, the selection of which being linked to plant phenology (temporal cycle of fruiting, flowering, and leafing in plants), availability, nutritional suitability, and spatial context (Olson 2011). Sufficient overlapping of hosts within their habitat, in conjunction with their well-developed flight capabilities (on average, flights were >0-1 km with a maximum recorded of 15.9km) (Babu *et al.* 2020) facilitates their dispersal through the environment, particularly after emerging from their overwintering sites when they tend to fly the furthest (Jones and Sullivan 1982, Pilkay *et al.* 2015, Tillman and Cottrell 2019, Babu *et al.* 2020).

1.4. Questioning the Subspecific Designation of the *Euschistus servus* Subspecies Complex

Blatchley (1926) asserts that a large series of *E. servus* specimens sampled from the entirety of the U.S. would show that the subspecific designation of *E. servus* is unwarranted given that they are just geographic races (*i.e.*, “part of a species marked by average differences in characters which intergrade with those of other subspecies occupying different, although usually adjacent parts of the general range of the species, along the common boundary of which intergradation is complete”). McPherson (1974) set a precedence for investigating *Euschistus* spp. under the suspicion that a particular *Euschistus* species complex exhibits plastic phenotypes that are subject to geographic variation (for a detailed synthesis on the patterns of geographic variation, causes, and compatibility with the subspecies concept, see Thorpe 1987) and should be considered polymorphic (a single species composed of multiple phenotypes that form freely interbreeding populations) as opposed to polytypic (species consisting of multiple subspecies).

Esselbaugh (1949), along with the support of R. I. Sailer, published a short paper noting that he was able to rear two distinct forms, *E. tristigmus pyrrhocerus* (Herrich-Schäffer) and *E. t. tristigmus* (Say), from a single parent collected from the intergrade zone between the two forms, thus supporting Blatchley’s earlier assertion that the two subspecies were environmentally inducible phenotypes, invalidating the then accepted subspecific designation. These forms violated the subspecific prerequisites of intergradation and geographic distribution. At the time, this assertion was not fully accepted because Esselbaugh had not standardized the light periodicity or the diet provided to the colony during rearing. Several decades later, McPherson (1974) supported Esselbaugh’s claims with experimental breeding data of his own. McPherson presented comparisons of offspring reared under two different photoperiods (constant light, 12 hours light and 12 hours dark), which resulted in a distinct dimorphism in adults within a single

population, thus supporting the notion that these two subspecies were instead morphs of a single species, suggesting that the phenotypes are plastic, and no longer supporting the subspecific designation of *E. tristigmus pyrrhocerus*.

Various localities may differ in any number of abiotic and biotic factors; for example, flora, fauna, soil composition, climate, precipitation, topography, etc. These factors exert selective pressures which may lead to variation amongst even the smallest geographic units. Geographic variation may influence the development of taxonomic differences, both actual and potential, among species, affecting many organismal characters. Those may include physiological and physical (size, proportion, coloration, pigmentation), internal structures, and cytological structures (Mayr 1942). In insects, general pigmentation (Gibert *et al.* 2004), genital armatures (Horton *et al.* 2016), epidermal, and chitinous structures (including exoskeleton anomalies) are often affected by geographic variation (Nikitin and Morozov 2016). Not all geographical variation is genotypical, and phenotypical changes of taxonomically important characters are common in insects (Mayr 1964). These differences are significant only if they have a genetic basis. Subspecific differences are generally due to a series of mutational steps and changes in chromosomal arrangements. Amongst a species complex, genetic differences should be more extensive than morphological and taxonomically important characters would indicate (Mayr 1964).

The *E. servus* subspecies complex has a broad continental distribution (Map 1) and may be susceptible to clinal variation. Clines occur most commonly within continental ranges where a continuous series of populations are found (Mayr 1964, Mayr and Ashlock 1991). As mentioned earlier (section 1.2.2.) Rolston (1974) concluded that *E. impictiventris* should be considered as a synonym of *E. servus*. He found that *E. servus* specimens exhibited a clinal shift in color of the

dorsum that changed according to Gloger’s rule of variation. Gloger’s rule notes that colors are more intense in humid and warmer places compared to colder and drier areas (Delhey 2017). We see an apparent inverse of Gloger’s rule in the antennal coloration between *E. s. euschistoides* (pale antennae with the 4th and 5th antennal segments dark) in the north and *E. s. servus* (antennae wholly pale yellow or with intense brownish red segments) in the south which may be explained by the previous established notion low temperatures and higher latitudes favors melanin development in arthropods (Dobzhansky 1933, Vernberg 1962, Rapoport 1969). Ultimately, when geographic variation within a species is clinal it should not be recognized as a subspecies (Mayr 1963, Mayr and Ashlock 1991). Unfortunately, the delineation of species at the lower limit is not so cut and dry and there have been arguments within the literature for over a century and half.

Table 1.4. Evidence for and against the subspecific designation of *E. servus*.

Subspecific Designation	Hybrid Zone	Variation	Diagnosable Differentiation	Phenotype
Valid	Yes	Discontinuous	Yes	Plastic
Invalid	Intergredation is complete	Continous and Clinal	Yes	Plastic

1.5. Reviewing the Subspecies Concept

The subspecies concept is one of the most controversial topics in systematics. The concept has been hotly debated and reviewed by a host of authors including many of the academic giants, some of whom have shifted their perspective (Keita 1993) as the debate has raged on. There have been several authors who have proposed different definitions and adjusted applications, where some have even suggested doing away with the trinomial system altogether, proposing the species as the terminal taxon (Wilson and Brown 1953, Gillham 1956, Terent’ev 1968 in Vinarski *et al.* 2015a), while another posits that the subspecies concept is a “tool for

convenience” and “genuine consensus on the topic is a pipe dream” (Fitzpatrick 2010). There are several other authors who have comprehensively summarized the debates and arguments for and against the subspecies concept over the past 150 years (Keita 1993; Winker 2010; Vinarski 2015a, b).

Within systematics there are three types of subspecies designations: 1) an original description at the subspecies level, 2) a formal rank reduction of a full species, and 3) an implied rank reduction of a full species. An implied rank reduction “results from the designation of a new subspecies that consequently reduces what becomes the nominate subspecies (Jorgensen *et al.* 2013).” Both *E. s. servus* and *E. s. euschistoides* were originally described as full species. *E. s. euschistoides* was later reduced to the subspecies level (*i.e.*, a formal rank reduction).

There has been a near eternal controversy over the definition and application of the widely used concept of a subspecies, particularly within Insecta (Remsen 2010). Wilson and Brown (1953) suggested the misuse of subspecies was due to complacency (*i.e.*, “I’ve found a variation, this must be a subspecies!”); they also speculated that many researchers did not sample enough individuals and based their conclusions on too few taxonomically significant morphological characters without considering the extent of geographical variation and disregarded sampling error (summaries of diversity change with increased sampling). Wilson and Brown (1953) further contended that allopatric populations must be gauged by genetics and morphology. They felt taxonomists may eventually abandon subspecific designations altogether, and that the different forms could be referred to by their geographic locality. Mayr (1953) railed against the use of subspecies in avian taxonomy. He noted that the subspecies category is not a concrete biological unit and should not be considered a taxonomically significant category similar to species, asserting that the subspecies concept is much more subjective. He further

suggested that it might be useful to utilize the subspecies concept, but it should only be utilized with greater specificity. Fox (1955) found the subspecies concept to be useful when applied consistently, but there is no consensus on how subspecies should be used. Even within the International Code of Zoological Nomenclature (ICZN, 2020a), there are several articles governing the application of the subspecies trinomen and any shift in nomenclature resulting from species name changes, but the ICZN does not provide a clear definition of what a subspecies is or how to delineate taxa at the lower taxonomic levels. Fox (1955) proposed that a satisfactory determination may be assessed when utilizing many specimens with a wealth of information. He noted that doing away with the subspecies concept would throw many areas of taxonomy into chaos and encouraged refining the criteria and "discouraging misuse" while arguing that past misuse does not invalidate the concept. In 1982, Mayr revisited his thoughts on the use of subspecies. He referred to it as a handle for taxonomic convenience but acknowledged that it was useful in distinguishing geographically separate populations. He further remarked that the subspecies concept fulfilled an important historical role by undermining the species concept and by contributing to a far better understanding of the geographic variation of species in nature. Mayr (1982) stated "the subspecies, despite its limitations and despite our occasional faulty applications, is a concept that has proved useful, and I think we will continue to use it." Even with its shortcomings, a well-defined subspecies may be used to discuss potential incipient species, refer to geographic subsets of species populations, note geographic variation in morphology (*i.e.*, designate clear morphological difference or designate clear color-pattern selection), and provide a foothold to understand distribution, diversity, and microevolutionary processes (Zusi 1982, Remsen 1984, Winker 2010, Hillis 2020).

1.5.1. Criticisms of the Subspecies Concept

What is a subspecies? There is no clear consensus on what a subspecies is or how it should be defined. However, there is a budding consensus that an integrative approach incorporating multiple lines of evidence should be utilized to identify and delimit subspecific taxa. These authors suggest that species delimitations should rely on more than one operational criterion utilizing all available data (Miralles *et al.* 2011, Hawlitschek *et al.* 2012, Torstrom *et al.* 2014, Patten and Remsen 2017, Galtier 2019). Miralles *et al.* (2011) published a comprehensive revision of Cape Verdean skinks (Squamata: Scincidae *Chioninia* Gray 1845) utilizing three lines of evidence 1) phylogenetic analysis utilizing existing published data, 2) genetic analysis using mitochondrial and nuclear data, and 3) morphological study based on scalation and color patterns. Hawlitschek (2012) noted that an integrative approach may still carry elements of arbitrariness because of the definitions of species and subspecies according to those lines of evidence. A common guideline, however, applied with generalized species concepts, common sense, along with relevant examples may yield more objective taxonomic descriptions. Many authors agree that working towards a fusion of phenotypical and genotypical data would lead to a more meaningful taxonomy (Miralles *et al.* 2011, Hawlitschek *et al.* 2012, Torstrom *et al.* 2014, Patten and Remsen 2017, Galtier 2019).

Why bother with subspecies? Despite the stated usefulness of the subspecies category, several authors have explicitly discussed the use and applications of subspecies (Smith and White 1956, Zusi 1982, Mayr 1982, Winker 2010, Hillis 2020) under both the biological species concept and the diagnosable and monophyletic phylogenetic species concept. Mayr (1964) defines a biological species as “groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups.” The diagnosable

monophyly based phylogenetic species concept is defined as “the smallest and diagnosable (diagnosable by a unique combination of character states in comparable individuals [Nixon & Wheeler, 1990]) cluster of individual organisms forming a monophyletic group within which there is a parental pattern of ancestry and descent” (McKittrick and Zink 1988). These alternative definitions both conceptualize species as separately evolving lineages (defined by Simpson [1961] as an ancestor-descendant series) through time, but they differ by what biological properties they are based on (*e.g.*, reproductive isolation and fixed character state differences) (de Queiroz 2007). Many authors agree that the concept’s practical applications lie in its usefulness in conservation decisions to “provide a criterion for prioritizing minimal units for conservation by ensuring that the evolutionary history (and potential) within species is maximized, protected, and maintained” (Braby *et al.* 2012) when the units reflect phenotypic and genetic differences (Braby *et al.* 2012, Thompson *et al.* 2020).

Subspecies are subject to sampling error. The plague of sampling error on subspecific designations was first recognized specifically by the 1st American Ornithological Union Committee on Classification and Nomenclature when formally adopting trinomialism (AOU 1886, in Winker 2010). Gillham (1956) noted one of the key objects of discontent was the arbitrary nature of the then applied subspecific designations. Gillham asserted that the boundaries drawn to separate continental subspecies at the time agreed with minimal character shifts, but rarely marked concordant change over broad distances. Wilson and Brown (1953) had hoped that the subspecific designation would benefit the study of geographic variation as opposed to perpetuating what they considered a subspecies mill. Noncritical subspecific designations serve to obscure the study of geographical variation and serve to clutter taxonomy with trinomens lacking validity (Vinarski *et al.* 2015a). Molecular based taxonomic studies are

not exempt from sampling error. This type of error also results from utilizing small molecular data sets and too few loci (Winker. 2010). The issue of sampling error has been widely resolved recently with diligent sampling. A larger sample size from a greater geographic area is critical to verifying initial diagnoses and describing new subspecies (Winker. 2010).

Can subspecies be a diagnosable biological unit? Mayr (1953) criticized the subspecies category for not being a concrete biological unit. Winker (2010) argued that subspecies can be diagnosable biological units. He further proposed that these diagnosable units should be diagnosable in the probabilistic (subject to or involving chance variation) sense using Amadon's 75% rule. The 75% rule stipulated that a subspecies may be valid if its individuals could be separated from nearly 100% of the overlapping population of individuals based upon their taxonomically significant characters (Amadon 1949). A probabilistic framework of diagnosability allows for the processes of divergence between populations and concurs with the biological species concept (*i.e.*, allows for hybridization) (Winker 2010). We would expect simple statistically significant differences, but they may not be grounds for the recognition of subspecies. Diagnosable subspecies facilitates research on dispersal, migration, local selection and adaptation, and biogeographic affinities (Patten and Unitt 2002, Winker 2010). Consistent application of statistical procedures such as Amadon's 75% rule will support the subspecies as a diagnosable biological unit.

Using diagnosability as a criterion, even with respect to the 75% rule, poses several problems. Winker 2010 stated: 1) Any prescribed level of diagnosability is arbitrary [Any p-value is an arbitrary level of statistical significance. Halsey *et al.* (2015) enumerated the issues with the p-value with regards to reproducible science]; 2) The outcome is driven by sampling size [*i.e.*, the closer to the diagnosability approaches our threshold, the more likely one individual

may change the entire taxonomic diagnosis without consideration to the biology and life history of the organism]; 3) The geography of sampling schema is critical to any outcome if the analyzed character shows any geographic variation; and 4) “Diagnosability is driven by the resolution of the technique used” [Mitochondrial DNA sequencing may be used to structure conspecific populations at a wide variety of evolutionary depths (Avice 2004)].

Subspecies pose a challenge to conservation. Haig and D’eila (2010) discussed how the subspecies concept influenced protections under the Endangered Species Act of 1973 (United States Government 1973), and how the continued use of the biological species concept versus the phylogenetic concept affected endangered species listing activities. Galtier (2019) also detailed the need for delineating species and the impact of salient species boundaries on policy. The Endangered Species Act allows the listing of species, subspecies, and “distinct population segments of vertebrates,” excluding “evolutionary units”. In addition, “significant portions of a species’ range” are eligible for protection. However, despite the language in the legislation, there is no consensus on what a “significant portion of a species range is, nor does it define population segments” (Haig and D’eila 2010). Subspecies lacking geographical specificity may mislead conservation policy as the subspecies concept may draw the attention of scientists and legislators alike who make decisions on the legal protection of the taxa and their conservation (Vinarski 2015b). Ambiguous language and scientific uncertainty make Endangered Species Act regulatory decisions difficult (Woods and Morey 2008). There is additional legislation implemented by the U.S. Fish and Wildlife Service to interpret and quantify the discreteness of a population segment (which is of importance to the taxon it belongs) (Haig and D’eila 2010). However, Woods and Morey (2008) found two error types in conservation decisions; that is, underconservation (defining too few taxa to effectively conserve biodiversity), and

overprotection (defining too many taxa). Underconservation may lead to loss of taxa and a lack of management actions before a species is critically endangered. Overprotection may lead to excessive costs and diminish conservation dollars (Haig and D'eila 2010). Additionally, Galtier (2019) noted that the distinct lack of a standardized species delimitation increased the arbitrary nature of lower taxonomic species delimitations by prodding researchers to willingly or unwillingly propose a taxonomy that concurs with their view on conservation, further harming research fields that rely on taxonomy.

There is a clear line between taxonomic categories and categories that are useful for other biological endeavors. Rigorous study of the world's species and exploring the intricacies of the web of life is not equivalent to conserving these species. One of the major issues being the two major kinds of conservation activities (listing and *in situ* actions) have different purposes, constraints and requirements (Mace 2004). For example, while federal agencies may utilize a subspecific designation to list an organism for protections, conservation biologists, historically, have utilized evolutionary significant units (while it traditionally included ecological and genetic data, it is not recognized as an acceptable taxonomic category) to help designate high priority for conservation and design the conservation and recovery plans for populations that merit separate management. Defining biologically meaningful units is critical when designing actions that should enhance species survivability (Crandall *et al.* 2000).

Without a doubt, taxonomy influences conservation. Managers assigning protection protocols must do so based on whether the organism is a true species, a populational subset of the species, or an ecotype. The legislation concerning threatened or endangered species specifies the name of the organism it intends to protect. But the protections protocols are not allocated to the organisms themselves; Endangered Species Act guidelines are allocated to the taxonomic

unit (*e.g.*, species, subspecies, etc.). Any change in the specific or subspecific designation could affect conservation measures. Thankfully the ICZN attempts to rectify this by protecting the names of endangered species (ICZN 2020b).

The relationship between conservation and taxonomy may seem obvious since conservation utilizes taxonomic categories to label which part of global biodiversity to conserve. This relationship depends on two widely accepted characteristics of this relationship: 1) conservation science is dependent on taxonomy, and 2) this relationship should not be interdependent to ensure subjective aspects of conservation does not inappropriately impact the descriptive undertaking of taxonomy (Conix 2019). However, this relationship has been contested with authors on both sides of the argument (*i.e.*, dependent versus interdependent relationship). Direct arguments can be seen in the 2017 papers between the proponents of a one-way dependent relationship for conservation and taxonomy (Raposo *et al.* 2017) and opponents who advocate that conservation science and taxonomy should be interdependent (Garnett and Christidis 2017). Conix (2019) reviewed these arguments and further argued that conservation science and taxonomy should be mutually dependent. He argued that conservation relied on taxonomy for an “inventory of biodiversity”, and taxonomy should rely on conservation to help resolve decisions through use of empirical and value-laden research.

How does the subspecies concept apply to species with low dispersal rates? Vinarski (2015a) stated that in a species with low vagility (degree to which an organism or taxon can or does move or spread within an environment), many highly isolated populations are microgeographical races that are easily distinguishable by taxonomic methods. For example, some mosquitoes (*Aedes* spp.) demonstrate clear color variations among species in between habitats. In East Africa, *Aedes aegypti* (L.) may be light colored within village huts and

earthenware, while darker forms breed outside in tree holes (Diehl and Bush 1984). Vinarski (2015a) posited that it would be “senseless” to describe each variant as a subspecies, despite the fact that these populations meet the subspecies criteria. In effect, this would create an endless number of subspecies and would be a taxonomic nightmare.

1.5.2. What is a Good Subspecies?

“Good” examples of subspecies recognize variation as discontinuous. Winker (2010) noted that there are plenty of examples of “good” subspecies which agrees with multiple definitions that in general define a subspecies as a species with recognizable populations exhibiting diagnosable differentiation from other related populations. For example, a “good” example of a diagnosable subspecies is the grasshopper genus *Chorthippus* Fieber. The *C. albomarginatus* (De Geer 1773) group of acridids in Europe are difficult to distinguish morphologically, but can often be distinguished by distribution, visual courtship display, calling song parameters, stridulatory peg dentition on the inside of the male hind femur, and antennal morphology (Vedenina and von Helversen 2009). Vedenina and von Helversen (2009) suggested that the suite of differences in the *C. albomarginatus* group of species was spurred on through rapid speciation via sexual selection, also noting that the divergence in sexual display and peg morphology promoted each other. This study resulted in the description of a new subspecies, *C. oschei pusztaensis* Vedenina and Helversen on the basis of peg morphology and calling song parameters, in addition to, they proposed the rank reduction of *C. bruttius* to a subspecies of *C. karelini*. These subspecies were described based on the suite of differences used to differentiate the *C. albomarginatus* group in addition to their recognizable populations throughout their geographic distribution. We may find examples of multiple character changes within many examples of taxa across multiple orders as well as species separated by hybrid zones (Barton and

Hewitt 1985, Vedenina and von Helversen 2009). We may also find that there are instances of ineffectual subspecies, but there are plenty of examples that satisfy their criterion and have the genetic and morphological evidence that satisfies their criterion for a valid subspecies (Mallet 2007).

1.6. Delineating Taxa Using Landmark Based Geometric Morphometrics.

Taxonomic workers have utilized several methods to evaluate a subspecies including traditional morphometrics (measurement and analysis of form) (Daly 1985), geometric morphometrics (Rohlf and Marcus 1993, Adams *et al.* 2014), genetics (Silva-Brandão *et al.* 2013, Hu and Wang 2019), various statistical analyses (*e.g.*, principal component analysis (PCA) and phylogenetic analysis) (Cook *et al.* 2006, Kato and Yagi 2008), and breeding (McPherson 1974). In some studies, a combination of methods were used. Barrientos-Villalobos *et al.* (2018) utilized a combination of landmark based geometric morphometric analysis and molecular analysis to determine that the differences in body shape of Mayan cichlid *Mayaheros urophthalmus* (Günther) within their study sites were consistent with ecophenotypic variation rather than genetic differentiation because of the geographic isolation. As a result, they concluded that the data did not support raising the traditionally recognized subspecies of *M. urophthalmus* (*i.e.*, *M. alborus* Hubbs, *M. cienagae* Hubbs, *M. conchitae* Hubbs, *M. mayorum* Hubbs, and *M. zebra* Hubbs) to the species level. As we attempt to investigate the subspecific designation of the *E. servus* subspecies complex, I have used the following subspecies definition as a basis: a subspecies is “an aggregate of phenotypically similar populations inhabiting a geographic subdivision of the range of a species and differing taxonomically from other populations of that species” (Mayr and Ashlock 1991). This investigation focused on using

landmark-based geometric morphometrics and multivariate statistical analyses (PCA and clinal analyses).

There are several papers that provide in depth analysis of these methods and the associated results (Rohlf and Marcus 1993, Adams *et al.* 2004, Sheets *et al.* 2006, Slice 2007), including their basic concepts and applications in entomology (Tatsuta *et al.* 2018). As taxonomy has long depended on comparative morphology, it is unsurprising that morphometrics (both the traditional and the relative newcomer geometric morphometrics [GM]) has proved to be so useful and increasingly used (Tatsuta *et al.* 2018).

There are distinct differences between traditional morphometrics and geometric morphometrics. Morphometrics is the study of covariances of biological form (Bookstein 1991), focusing on the description and statistical analysis of geometry within organisms at various scales. Traditional morphometrics or multivariate analysis is characterized by using linear measurements taken from morphological features (*i.e.*, lengths, widths, angles, ratios etc.) to capture shape, and analyzed with PCA, factor analysis, Canonical Variates Analysis (CVA), and discriminant function analysis. Unfortunately, allometry (change in organisms in relation to proportional changes in body size), the inability to capture complete shape data, and the inability to assess size and shape separately (Demayo *et al.* 2007) has proved to be a major problem with these analyses. Geometric morphometrics uses coordinates of points called landmarks in lieu of angles and distances. These landmarks are superimposed through translation, scaling, and rotation. This superimposition results in the landmark data differing only in shape which is subsequently analyzed by multivariate statistical methods (similar methods used in traditional morphometrics) (Tofilski 2008). With the advent of GM and the use of semi-landmark data, outline data, and landmark data scientists can capture complete shape data accounting for issues

of size (Rohlf and Marcus 1993, Adams *et al.* 2004, Slice 2007). Geometric morphometrics has been used widely through various biological science and repeatedly to delineate taxa within entomology (Mutanen and Pretorius 2007, Ludoški *et al.* 2008, Jaramillo-o *et al.* 2015, Chaiphongpachara *et al.* 2019, Sim and Zuha 2019)

GM has been applied to various structures amongst various taxonomic groups (genus, subgenus, species, and subspecies) (Aytekin *et al.* 2007, Tofilski 2008, Mondal *et al.* 2015, Li *et al.* 2017), populations, spatial scales (scope of an area at which a process or phenomenon occurs) (Diniz-Filho *et al.* 2000, Sadeghi *et al.* 2009), and morphoforms (morphological form of an organism) (De Souza *et al.* 2015) within entomology (Tatsuta *et al.* 2018, Henriques *et al.* 2020) which have included new species descriptions (Da Rosa *et al.* 2012, Dorn *et al.* 2018). For example, wing venation has been analyzed frequently with geometric morphometrics in several orders of Insecta (Coleoptera, Hemiptera, Lepidoptera, Odonata, Orthoptera, and especially Diptera and Hymenoptera) (Tatsuta *et al.* 2018). Geometric morphometrics has been used in a relatively novel manner to delineate ant species (Hymenoptera: Formicidae) by analyzing male genital morphology within the genus *Dinoponera* Roger to help clarify specific level relationships including cryptic species (Tozetto and Lattke, 2020). Geometric morphometrics has also been used to delineate infraspecific variation in Hemiptera (Torres *et al.*, 2010; Sepe and Demayo 2014, 2017; Vilaseca *et al.*, 2020).

Landmark-based GMA has been used in the Pentatomidae to ascertain variation in populations (Demayo *et al.* 2007, Cruz *et al.* 2011, Torres *et al.* 2013, Sepe and Demayo 2017), sexual dimorphism (Sepe and Demayo 2014), to investigate a quantitative hypothesis driven approach to describe species (Fernández-Aldea *et al.* 2014), and to resolve species conflicts within a genus (Li *et al.* 2017). Most of the GM investigations within the Pentatomidae has been

within the genus *Scotinophara* Stål (Demayo *et al.* 2007; Cruz *et al.* 2011; Torres *et al.* 2013; Sepe and Demayo 2014, 2017) with only a couple of studies on other genera (Fernández-Aldea *et al.* 2014, Li *et al.* 2017). These studies utilized landmark-based GMA to ascertain differences within head, pronotum, hind wing shape, and wing venation to determine variability within rice black bug (*Scotinophara* spp.) populations, or sexual dimorphism as influenced by geographical or other abiotic factors (*e.g.*, rice host varieties). For example, Sepe and Demayo (2014) used landmark-based GM to validate the existence of sexual dimorphism within *Scotinophara coarctata* Stål by assessing the shape of the head from a dorsal view, and then identifying discrepancies between sexes. The authors found that CVA revealed discrete groups and thin-plate spline visualizations revealed sexual dimorphism. Further, they noted that the tylus and other midline structures of the head projected left or right. They tendered that the dimorphism may be associated with the increased feeding linked to egg production. Additionally, they suggested that the differences between populations may correspond to the genetic diversity of host plants and the genetic variability of the insects feeding on those plants.

Apparently, there have been no investigations utilizing geometric morphometrics to delimit species or subspecies within the genus *Euschistus*. Geometric morphometrics is a powerful low-cost tool (Henriques *et al.* 2020) that has been used to analyze and characterize infraspecific variation across various spatial scales making it a relevant tool to provide a line of evidence to investigate subspecific designations.

1.7. The Current Study

Blatchley (1926) speculated that a wide-ranging geographic sample of *E. servus* specimens from the entirety of the U.S. would show that the subspecific designations within the *E. servus* subspecies complex would probably show that these designations were unwarranted.

The current study seeks to reevaluate the subspecific designation of *E. servus* utilizing landmark-based GM and multivariate analysis. Our objective was to determine whether *E. servus* subspecies and intergrade populations express distinct suites of taxonomically important morphological characteristics throughout the United States. Given that *E. servus* has an extensive distribution throughout the contiguous North America, this makes them ideal for assessing potential variation in their taxonomically significant morphological characteristics and determining the pattern of geographic variation their phenotypes may exhibit. Ultimately, this research aims to contribute towards developing to a unified subspecies concept, and to strengthen an integrative approach to delineating taxa at the lower taxonomic levels.

1.8. References

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**CHAPTER 2. INVESTIGATING THE SUBSPECIFIC DESIGNATION OF THE
BROWN STINK BUG, *EUSCHISTUS SERVUS* (SAY) (HEMIPTERA: HETEROPTERA:
PENTATOMIDAE), USING A COMBINED LANDMARK- BASED GEOMETRIC
MORPHOMETRICS APPROACH**

2.1. Abstract

Euschistus servus (Say, 1831) (Heteroptera: Pentatomidae) is distributed throughout North America and is currently subdivided into two subspecies *E. s. servus*, found in the southern U.S., and *E. s. euschistoides* (Vollenhoven, 1868) in the northern U.S. An intergrade population inhabits the central U.S. from Maryland to Kansas. This study sought to reevaluate the established subspecific designations and determine whether *E. servus* subspecies and intergrade populations express distinct suites of taxonomically important morphological characteristics throughout the U.S., or if the characteristics exhibit clinal variation. We quantified landmark-based geometric morphometric data in conjunction with traditional morphometrics (*i.e.*, relative lengths of the juga and tylus) from specimens collected along a wide geographic area and used a principal component analysis to determine if there is a significant difference within the subspecies' morphology. Principal component analysis assessing shape between morphogroups failed to produce clusters commensurate to the *E. s. servus* and *E. s. euschistoides* forms. Analysis of the shape, connexivum, and antennal color characters suggested that there is linear relationship between the taxonomic characters and latitude as the predictor and explanatory variable which may be indicative of clinal variation. This data leads us to dispute the existing subspecific designation of the *E. servus* species complex. We believe *E. servus* should be recognized as polymorphic as opposed to polytypic.

2.2. Introduction

Members of the species *Euschistus servus* (Say 1831) are voracious polyphages that feed on many different host plants, including economically significant crops and weeds (Greene et al. 2001). This stink bug species is distributed throughout North America and is currently subdivided into two subspecies, *Euschistus servus servus* which is found primarily south of 40° latitudes, and *Euschistus servus euschistoides* (Vollenhoven 1868) found north of 40° latitudes. There is also an intergrade population, which exhibits intermediate morphological characteristics, stretching from Maryland to Kansas (Fig. 2.2). Adult specimens (both sexes) in both subspecies range from 11.0 to 15.0 mm in length (*i.e.*, from the apex of the genital segment to the tip of tylus). The two subspecies are distinguished primarily by differences in the color of the antennal segments, more or less exposure of the connexiva, and by differences in relative lengths of the juga and tylus. *Euschistus s. euschistoides* specimens generally have juga that are distinctly longer than the tylus, antennal segments 4 and 5 are usually dark in color, and the connexiva is usually completely covered by the hemelytra. In contrast, *E. s. servus* specimens generally have juga that are subequal in length to the tylus, the antennal segments are more uniformly colored yellowish or reddish brown, and the connexiva is usually broadly exposed. The intergrade population is recognized as having characteristics intermediate between those listed above (Fig. 2.1) (Sailer 1954, McPherson 1982).

McPherson (1974) set a precedence for investigating *Euschistus* spp. under the suspicion that a particular *Euschistus* species complex exhibits plastic phenotypes that are subject to geographic variation and should be considered polymorphic as opposed to polytypic. McPherson presented comparisons of offspring reared from two distinct forms of *E. tristigmus* (Say): the nominate subspecies and *E. tristigmus pyrrhocerus* (Herrich-Schäffer). He found that the

offspring from a single parent collected from the intergrade zone between the two forms, when reared under two different photoperiods (constant light, 12:12 L:D), resulted in a distinct dimorphism in adults within a single population. This supported the notion that these two subspecies were actually morphs of a single species, suggesting that the phenotypes were plastic. Thus, these results did not support the subspecific designation of *E. tristigmus pyrrhocerus*. Both *E. s. servus* and *E. s. euschistoides* were originally described as full species. *Euschistus s. euschistoides* was later reduced to the subspecies level (Sailer 1946). Blatchley (1926) speculated that a wide-ranging geographic sample of *E. servus* specimens from the entirety of the U.S. would show that the subspecific designations within the *E. servus* subspecies complex is unwarranted.

Traditional morphometrics is characterized by using linear measurements taken from morphological features (*i.e.*, lengths, widths, angles, ratios, etc.) to capture shape. Unfortunately, allometry (change in organisms in relation to proportional changes in body size), the inability to capture complete shape data, and the inability to assess size and shape separately (Demayo *et al.* 2007) has proved to be a major problem for traditional morphometrics. Geometric morphometrics uses coordinates of points called landmarks in lieu of angles and distances and is analyzed with similar multivariate statistical methods (*i.e.*, PCA, factor analysis, canonical variates analysis (CVA), and discriminant function analysis). With the advent of GM and the use of semi-landmark data, outline data, and landmark data, scientists can capture complete shape data accounting for issues of size (Rohlf and Marcus 1993, Adams *et al.* 2004, Slice 2007). The fact that *E. servus* has an extensive distribution throughout North America makes this species ideal for assessing the variation in taxonomically significant morphological characteristics and determining the pattern of geographic variation their phenotypes may exhibit. These conditions

encourage the use of a combination of traditional and geometric morphometrics to potentially identify the type of geographic variation exhibited among *E. servus* populations.

The decision to choose landmark-based geometric morphometrics (GM) versus outline-based GM varies depending on the species chosen for any study, both perform comparably and are able to reasonably distinguish between groups. The landmark-based GM is advantageous because it demands less time, requires fewer coordinate points for analysis, and requires fewer samples (Chaiphongpachara 2018). Geometric morphometrics is a powerful tool that has been used to analyze and characterize infraspecific variation across various spatial scales (Henriques et al. 2020) making it a relevant tool to provide a line of evidence to investigate subspecific designations. There are no investigations utilizing landmark-based geometric morphometrics to delimit species or subspecies within the genus *Euschistus* (Masonick and Weirauch 2019, Henriques et al. 2020., Kamimura et al. 2020).

The present study seeks to reevaluate the subspecific designation of *E. servus* using traditional morphometrics, landmark-based GM, and multivariate analysis to determine whether *E. servus* subspecies and intergrade populations express distinct suites of taxonomically important morphological characteristics throughout the U.S. With the geographic distribution, morphology, and previous work done on *Euschistus* in mind, we asked two questions: 1) Are the taxonomically significant morphological characters of the *E. servus* significantly different when measured between morphological forms?, and 2) Does the taxonomically significant morphological characters of the *E. servus* subspecies complex exhibit clinal variation within a north-south or south-north latitudinal gradient?

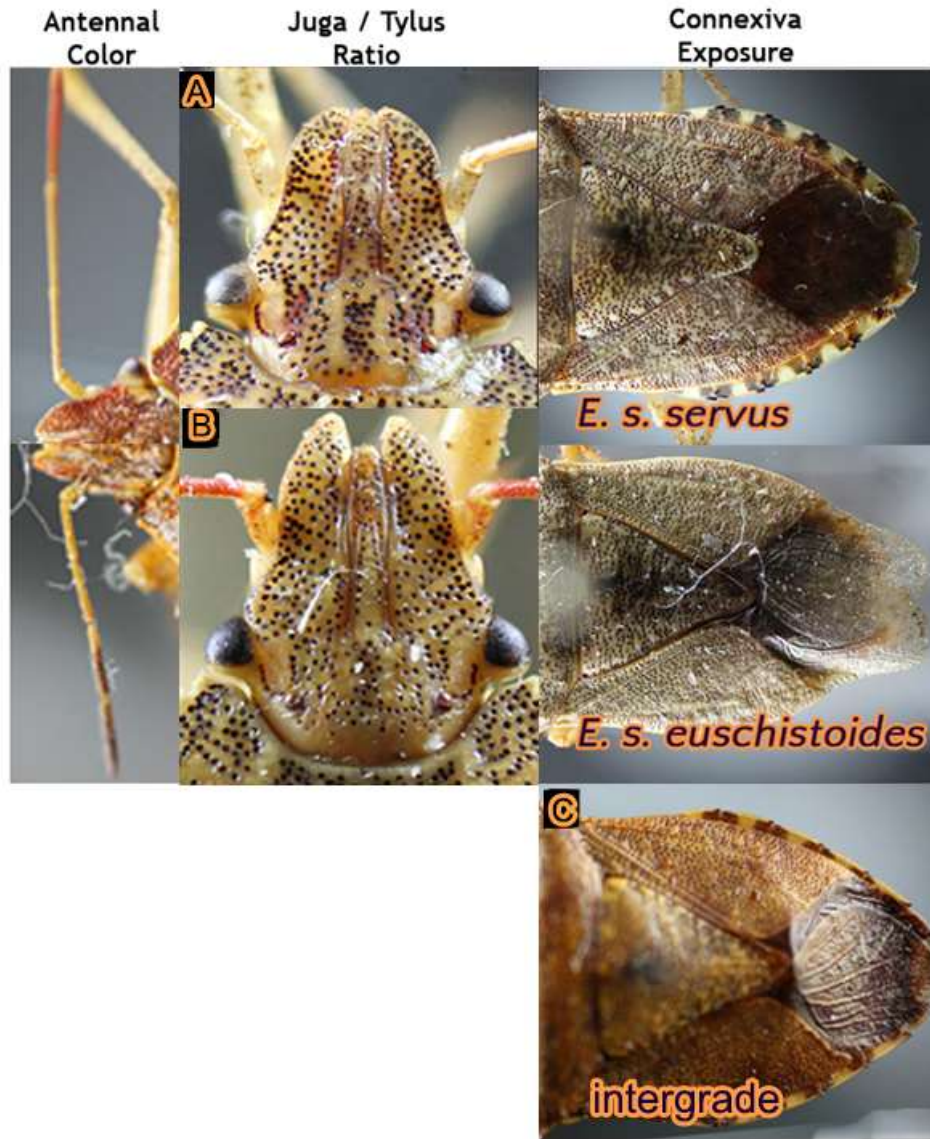


Figure 2.1. Morphological differences between the A) *E. s. servus*, B) *E. s. euschistoides* C) intergrade forms.

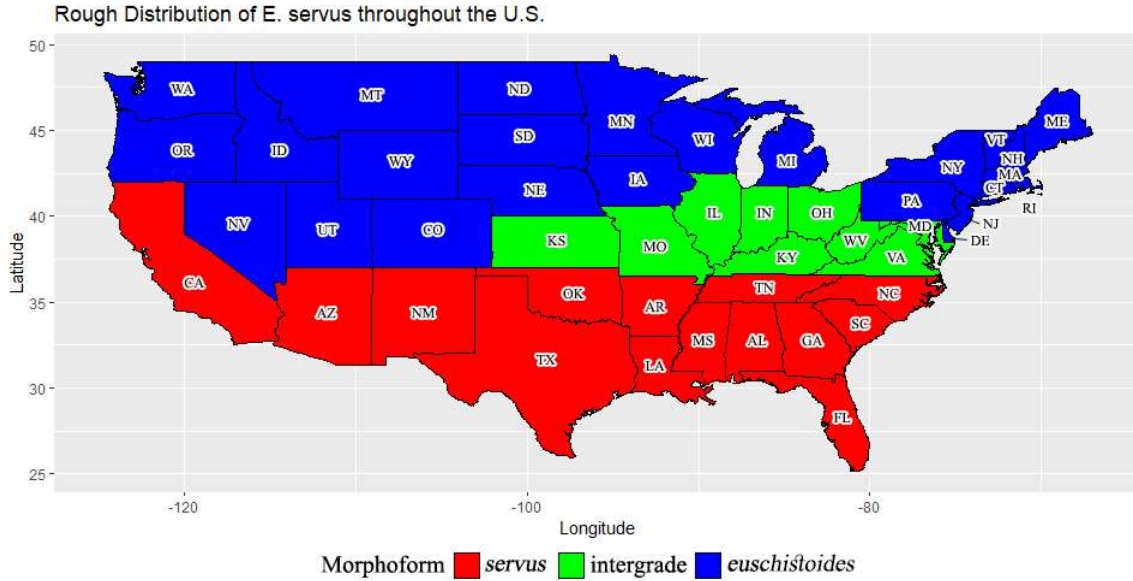


Figure 2.2. Distribution of *E. servus* subspecies throughout the United States (modified from McPherson 1982).

2.3. Materials and Methods

2.3.1. Specimens

Euschistus servus specimens ($n=768$) used in this study were borrowed from the following institutions: North Dakota State Insect Research Collection (Fargo, ND, Dr. David A. Rider, 170), University of Connecticut Biodiversity Research Collections (Mansfield, CT, Dr. Janine Caira, 27), University of Minnesota Insect Collection (St. Paul, MN, Dr. Robin Elizabeth Thompson, 48), University of Missouri Enns Entomology Museum (Columbia, MO, Dr. Kristin B. Simpson, 116), University of Nebraska State Museum (Lincoln, NE, Dr. Brett Ratcliffe, 61), and Washington State University M. T. James Museum (Pullman, WA, Dr. Richard Zack, 97). For each collection, we selected a random sample of *E. servus* adults, and from those chose mature, non-degraded specimens to use in our analysis to ensure accurate measurements.

2.3.2. Locality Data

Locality data was extracted from the labels attached to the acquired specimens. Geographic coordinates of all specimen localities was determined to the nearest 0.1 of latitude and longitude (decimal degrees) using data from Geographic Names Information System (GNIS) developed by the United States Geological Survey (USGS). Specimens borrowed from museums were originally collected between latitudes 25° and 48° and for localities (or small regions) that contained multiple individuals. Latitude is used as one the primary independent variable for the statistical analyses because the *E. servus* subspecies complex is found to vary in from the southernmost latitudinal plane of the U.S. to the northernmost latitudinal plane. In doing so, we may not capture other integral parameters that may determine morphology (*e.g.*, altitude).

2.3.3. Study Area

The study area chosen in this study is the contiguous U.S. (Fig. 2.3. and Fig. 2.4.). The U.S. is an ecological mosaic ranging from mountainous forests, to deserts, to taiga, to plains, to tropical wet forests. In general, the climate becomes colder the further north and more humid the further east (except for the western coast). The variety of climate zones vary due to the varied geographic features, changes in latitude, and altitudes (Beeman et al. 2021). This impacts the distribution of flora, fauna, and abiotic conditions.

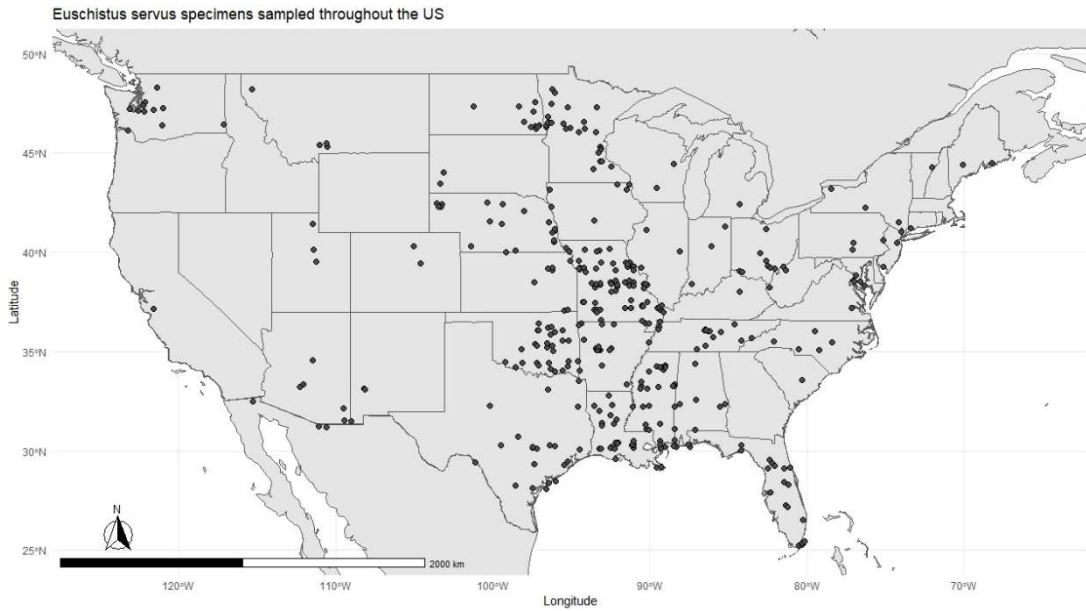


Figure 2.3. Map of the contiguous USA depicting sample points of our specimens used in the analysis ($n=768$).

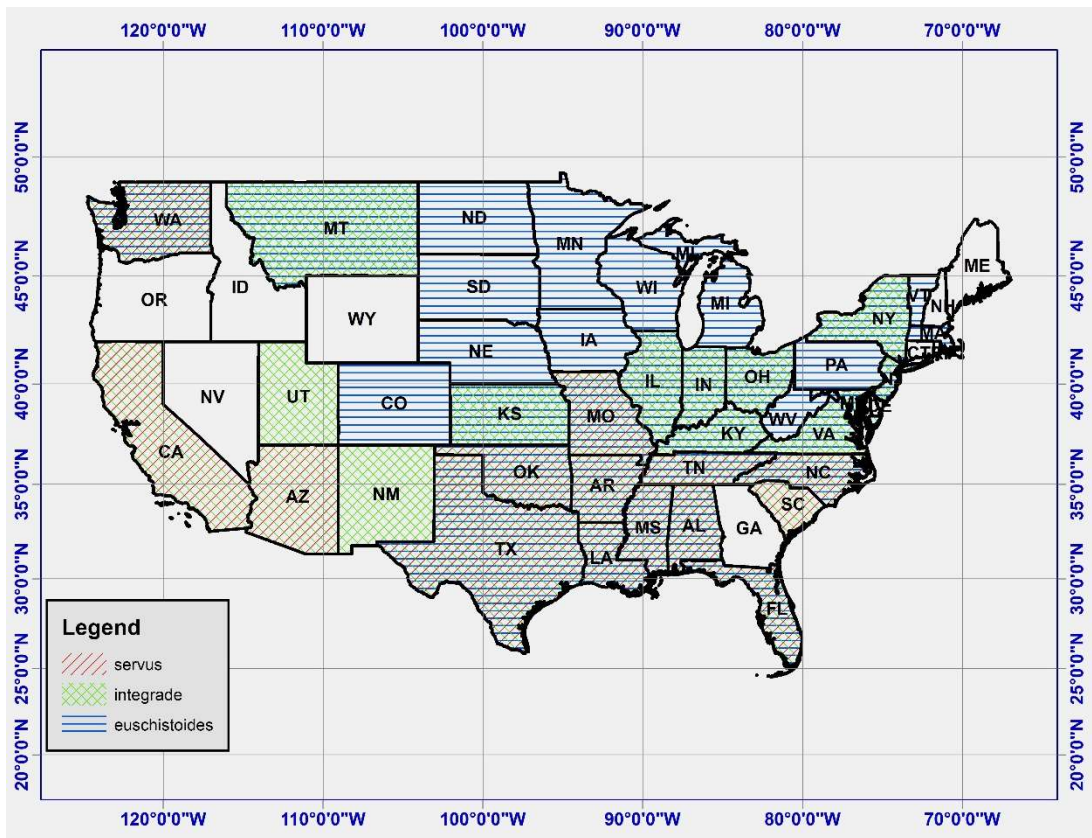


Figure 2.4. Distribution of *E. servus* based on the data collected, for states that are white, no data specimens were examined from that state.

2.3.4. Morphology Assessment, Specimen Digitization, and Statistical Analyses

We focused on landmark-based geometric morphometrics characterizing dorsal head shape in addition to the taxonomically significant physical characteristics used to delineate *E. servus* subspecies: 1) exposure of the connexiva segments, 2) color of the antennal segments IV and V, and 3) the relative length of the juga and tylus (McPherson 1982).

Specimens were identified as 1-3 (Table 2.1) based on the composition of their morphological characteristics prior to taking photos. If a specimen had a combination of characters (*e.g.*, broadly exposed connexivum, antennal color entirely pale or red, and relative jugal lengths subequal to tylus) it was classified as intergrade.

Table 2.1. Characteristics used to categorize *E. servus* morphoforms.

Score	Form	Connexiva	Antennal Color	Relative Juga versus Tylus Length
1	<i>E. s. servus</i>	Broadly exposed	pale, red, or brown	Equal to subequal
2	intergrade	Any or partially exposed	any	Any
3	<i>E. s. euschistoides</i>	Completely covered	IV-V antennal segments brown	Greater than

Photos of specimens for the traditional and geometric morphometric analysis were taken with a customized set up consisting of a Canon EOS 7D Mark II digital single-lens reflex camera (CANU9, Tokyo, Japan) fixed to a CopyStand CS-1070 situated above a StackShot™ automated macro rail designed for focus stacking with a fixed stage. All specimens were photographed from a fixed distance to reduce any error introduced from changes in pixel size.

2.3.4.1. Connexiva and Antennal Color

The condition of the connexiva was scored as follows: connexiva completely or nearly completely exposed in dorsal view (1); connexiva partially exposed in dorsal view (2); and connexiva nearly or completely covered from dorsal view (3). The color of antennal segments IV

and V range from dark brown, or entirely dark brown (fuscous) to pale or reddish. This was scored from 1-3 with 1) antennae entirely pale, 2) antennae entirely fuscous to red, and 3) antennal segments IV and V being dark brown. We conducted a multinomial logistic regression to determine whether independent variable, latitude, significantly predicted the variation within the outcome variables, connexivum and antennal color, respectively to analyze the findings and assess potential clinal variation.

2.3.4.2. Traditional Morphometrics: Relative Length of Juga and Tylus

A photo of each specimen was imported into ImageJ. The measurements of the juga and tylus were standardized by taking the length (mm) from an imaginary transverse drawn through the posterior margins of the ocelli (present on all specimens) to the apices of the juga or tylus (Fig. 2.5). This distance was converted into physical distance with the use of the ruler function within ImageJ, based on a scale created by using the original images taken with a ruler (*i.e.*, we calculated that 2018 pixels = 0.5 cm based on an image with the same resolution) to obtain the relative lengths of each jugum and tylus. The relative lengths of the juga were averaged prior to being compared with the tylus to calculate the differences in length. We used traditional morphometrics to provide evidence to substantiate the subspecific designation of *E. servus* in addition to support for our GM findings and assess potential clinal variation in the head shape of *E. servus*. Histograms of the data demonstrated a normal distribution for both sets of variables. We used linear regression using latitude as the independent variable to determine any significant relationship between the response variables; relative differences in length of juga and tylus, and the relative length of the jugal average. An ANOVA was conducted between morphoform (factor with 3 levels) to determine any difference between in relative difference in length of juga and

tylus, and relative length of average juga. If the ANOVA was significant, we conducted a post-hoc comparison using the Tukey HSD test.

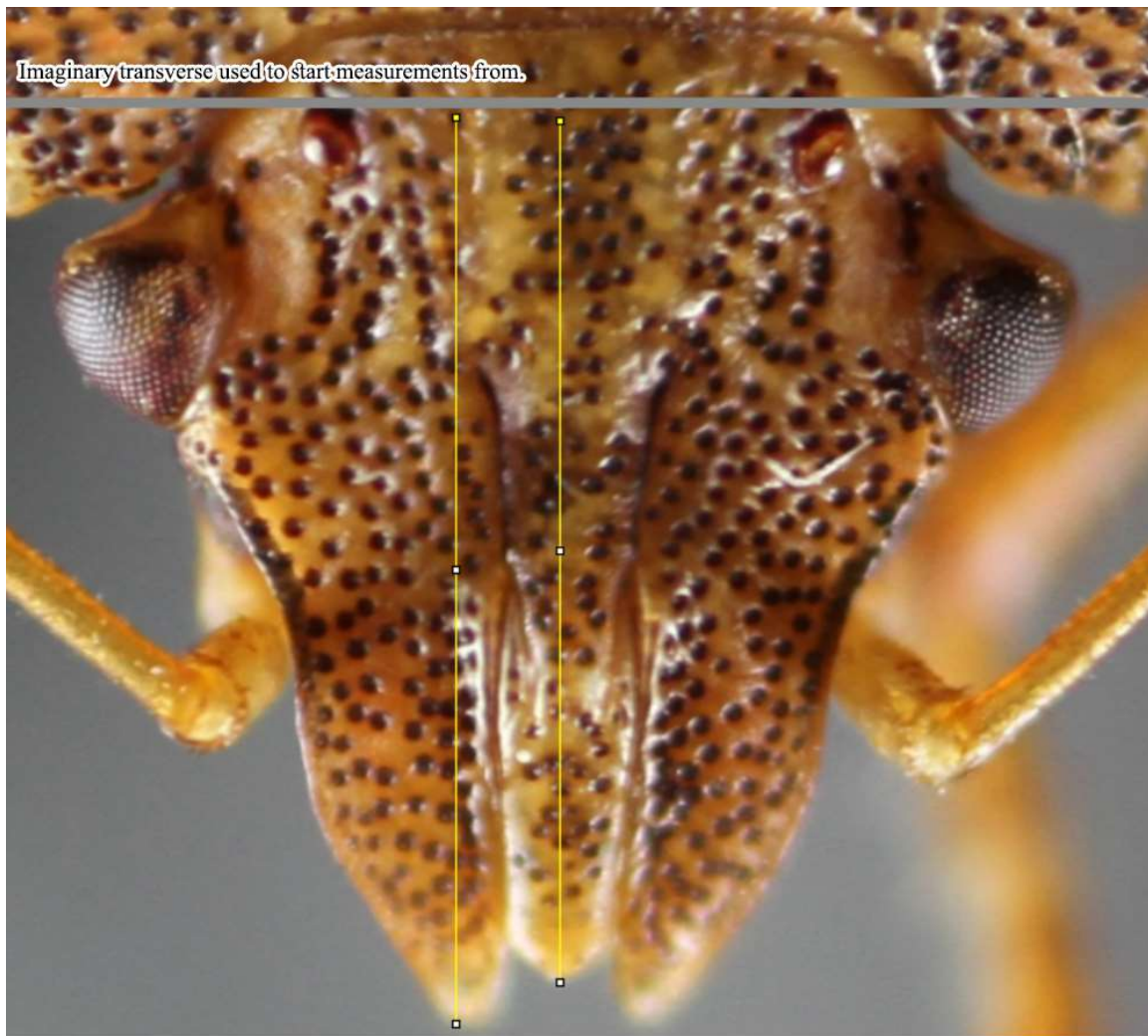


Figure 2.5. Imaginary transverse placed at the posterior margins of the ocelli used to calculate the relative lengths of the juga and tylus.

2.3.4.3. Geometric Morphometrics: Head Shape

Each specimen's image was imported to build a .tps file in tpsUtil version 1.79 (Rolf 2019). Specimens were then digitized (landmark placement) in the TpsDig version 2.32 software (Rolf 2018). This file was then imported into R studio workspace where the dorsal view of the

head was analyzed using the geomorph package (Collyer and Adams 2018) and the data was run through multiple multivariate analyses.

The cartesian coordinates of nine landmarks on the head (Fig. 2.6, Table 2.2.) were chosen based on symmetries exhibited throughout the species' head morphology (e.g., tip of the juga, location of compound eyes, and ocelli). The landmarks selected were based upon previous work (Sepe and Demayo 2014) then altered to maximize the capture of points that allowed for analysis of differences in the ratio of juga/tylus, since this is one of the most taxonomically significant characteristics of the species. Selection of the landmarks adhered to the requirement that they are easily and repeatedly found on the organism (Bookstein 1991) across all specimens. We controlled for any potential "user effect" (persistent differences that may arise between different users in pointing to a location of some landmarks that impacts final output) (Dujardin 2014) by having a single user collect landmark data.

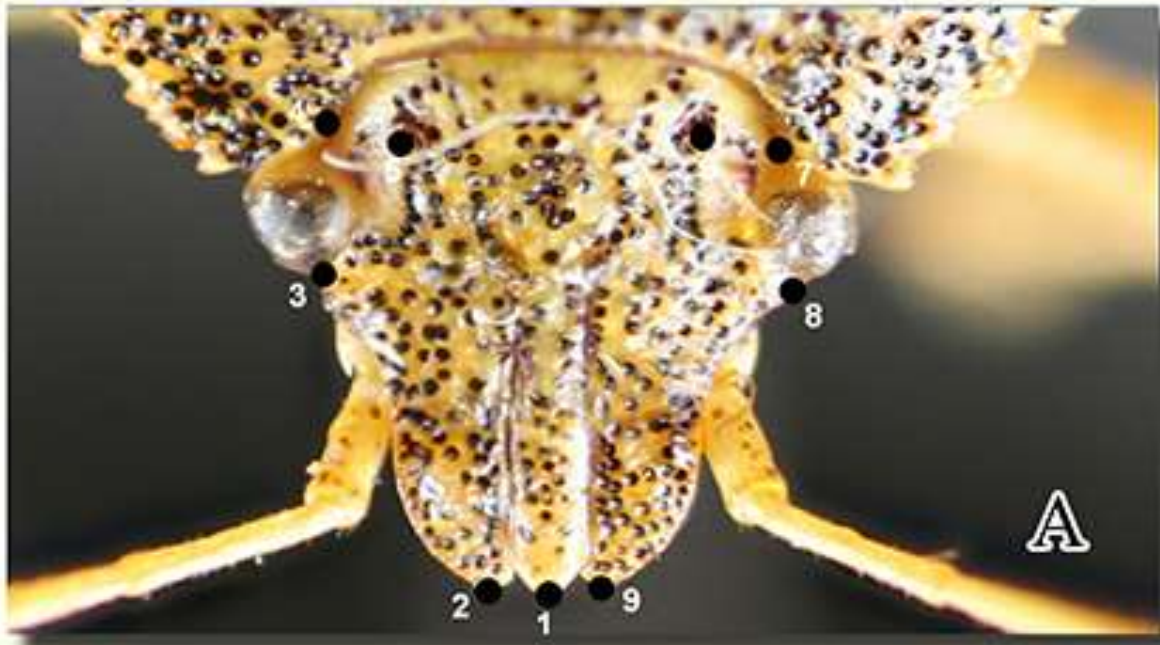


Figure 2.6. Position of the nine digitized landmarks on the head (dorsal view) of *E. servus*.
 A) Example of subequal - equal relative length of juga and tylus B) Example of greater relative length of juga and tylus.

Table 2.2. Description of digitized landmarks of *E. servus* specimens

Landmark Number	Description
1	Apical point of tylus
2, 9	Apical point of juga
3, 4, 7, 8	Position of compound eyes
5, 6	Position of ocelli

We used generalized Procrustes analysis in R (version 3.6.3) to calculate eigen values for each principal warp and obtained shape variables from the landmark data (Rolf and Slice 1990). Generalized Procrustes analysis translated the landmark coordinates using a least-squares criterion and calculated (via scaling and rotating each point to the origin of the cartesian plane) a unit-centroid size to define the shape of each specimen (Tatsuta et al. 2018). The centroid size is a measurement used explicitly within GM. It is calculated by minimizing the sum of the distance between corresponding landmarks of an object from their center of gravity (*i.e.*, centroid, obtained by averaging x and y coordinates from all landmarks). The centroid size translates to the discrepancy between landmarks that is not removed by scaling, translation, or rotation. This quality makes it useful as a measurement of shape difference (Klingenberg 2016, Tatsuta et al. 2018).

The first step of data analysis in a GM study (Fig. 2.7), the PCA is a statistical technique in which a large set of data from multiple populations are represented as 2 or 3 dimensional points on a cartesian plane. The PC analysis based upon the nine head landmarks yielded 18 principal components (one dimension for each x and y coordinate per landmark). Principal component analysis was conducted to analyze shape variations of the Procrustes aligned specimens' centroid size within and among morphogroups in R using the autoplot function of the ggfortify package (Tang et al. 2016). A scatterplot (morphospace) of the first two principal components was generated to graphically summarize the distribution of individuals and centroid

size within morphogroups. The resulting morphospace yielded points on the plot that represented a different configuration of landmarks. Thin-plate splines (TPS) deformation grids were utilized to graphically depict shape change based on landmarks along PC axes compared to the mean shape. We also conducted a non-parametric Kruskal-Wallis Test to examine the differences of centroid size, since the data was bimodally distributed, according to the morphoform recorded.

2.3.5. Statistical Assumptions

Each of the statistical tests we performed were based on a set of assumptions. Linear regression and one-way ANOVA shared several key assumptions. Both tests assumed homoscedasticity, independence of observations, and normality, and the dependent variable being continuous. Linear regression and PCA both assumed linearity in the data set. Kruskal – Wallis test and Multinomial logistic regression also assumed independence of observations. Kruskal – Wallis tests also assumed random sampling and homoscedasticity.

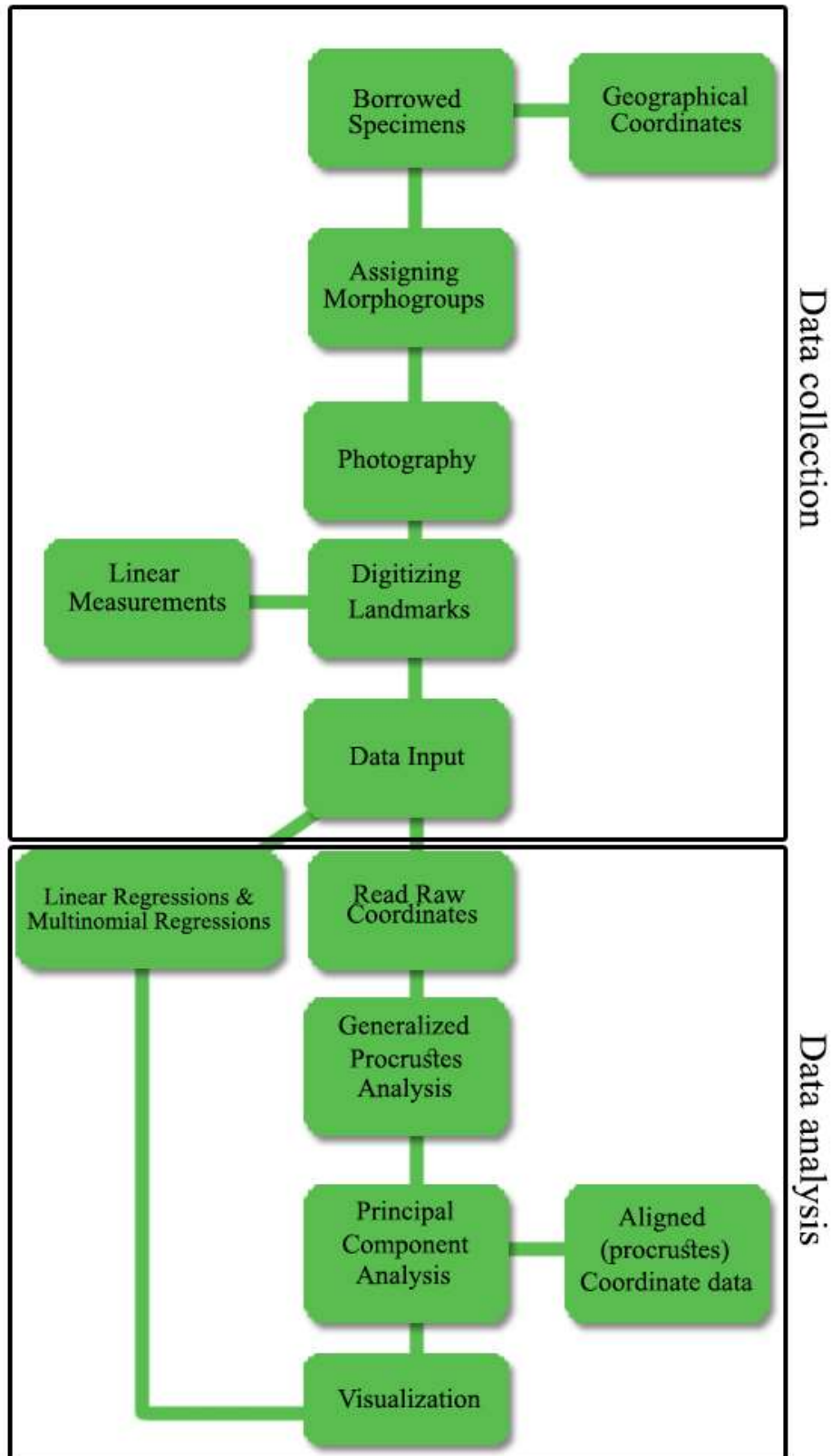


Figure 2.7. A simplified graphical workflow representing the methodology of the study.

2.4. Results

2.4.1. Traditional Morphometrics: Connexivum and Antennal Color

When assessing the model fit information (Fig. 2.8) for the latitude predicting connexivum state, the log-likelihood indicated that there was a difference of 246.55. The likelihood ratio of chi-square of 246.55 with a p-value < 0.0001 means that the model explains a significant amount of the original variability and fits significantly better than the null model (*i.e.*, model with no predictors). When assessing the model fit information for the latitude predicting color state (Fig 2.9), the log-likelihood indicated that there was a difference of 140.22. The likelihood ratio of chi-square of 140.22 with a p-value < 0.0001 means that the model explains a significant amount of the original variability and fits significantly better than the null model. These models suggest that as latitude increases, the probability of finding the northern *euschistoides* suite of characteristics (completely covered connexiva, and antennal segments IV-V entirely brown) increases. Conversely, as the latitude decreases, the probability of finding the southern *servus* suite of characteristics (connexivum completely exposed, antennal color more uniformly pale yellow or dark brown or red) increases. This translates to *E. servus* are more likely to express *euschistoides* forms above 45 degrees latitude.

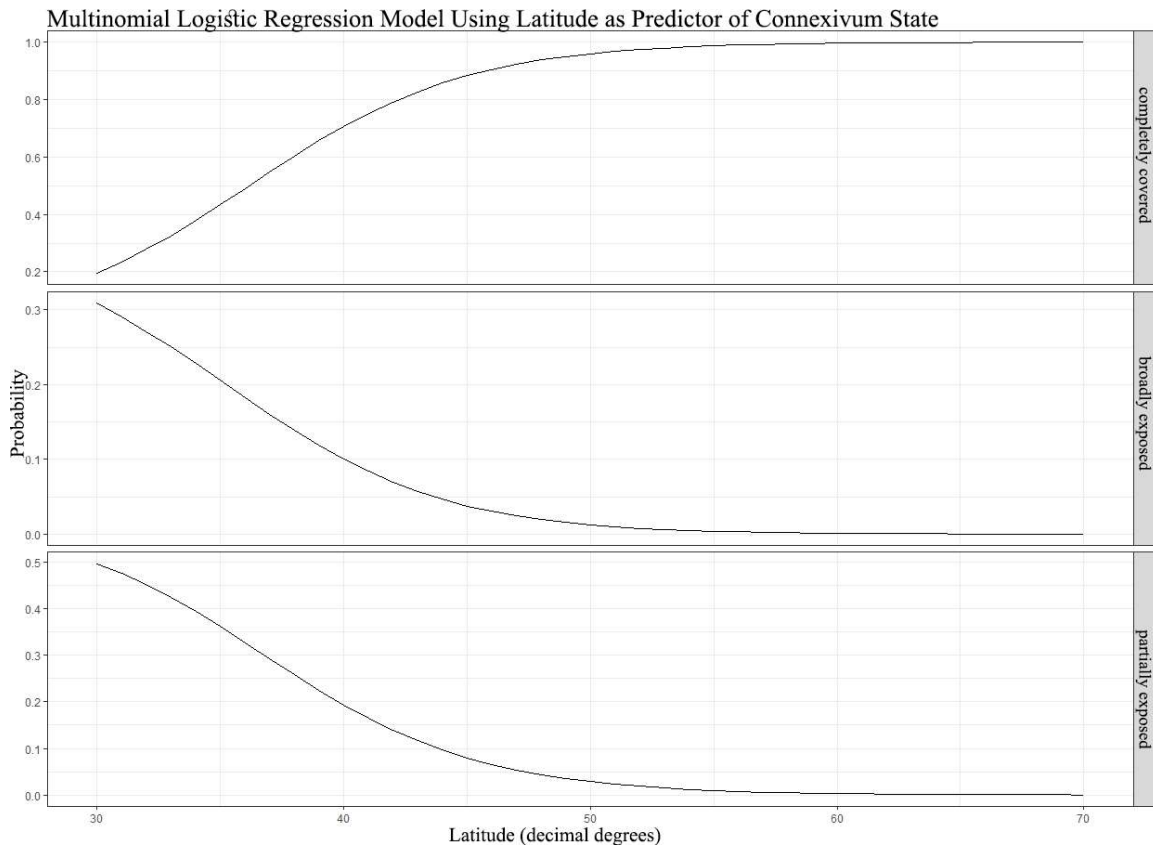


Figure 2.8. Multinomial linear regression of the predictor latitude on connexival state demonstrating the relationship between the two variables. Likelihood ratio tests validate the use of latitude as a predictor versus a model with no predictor.

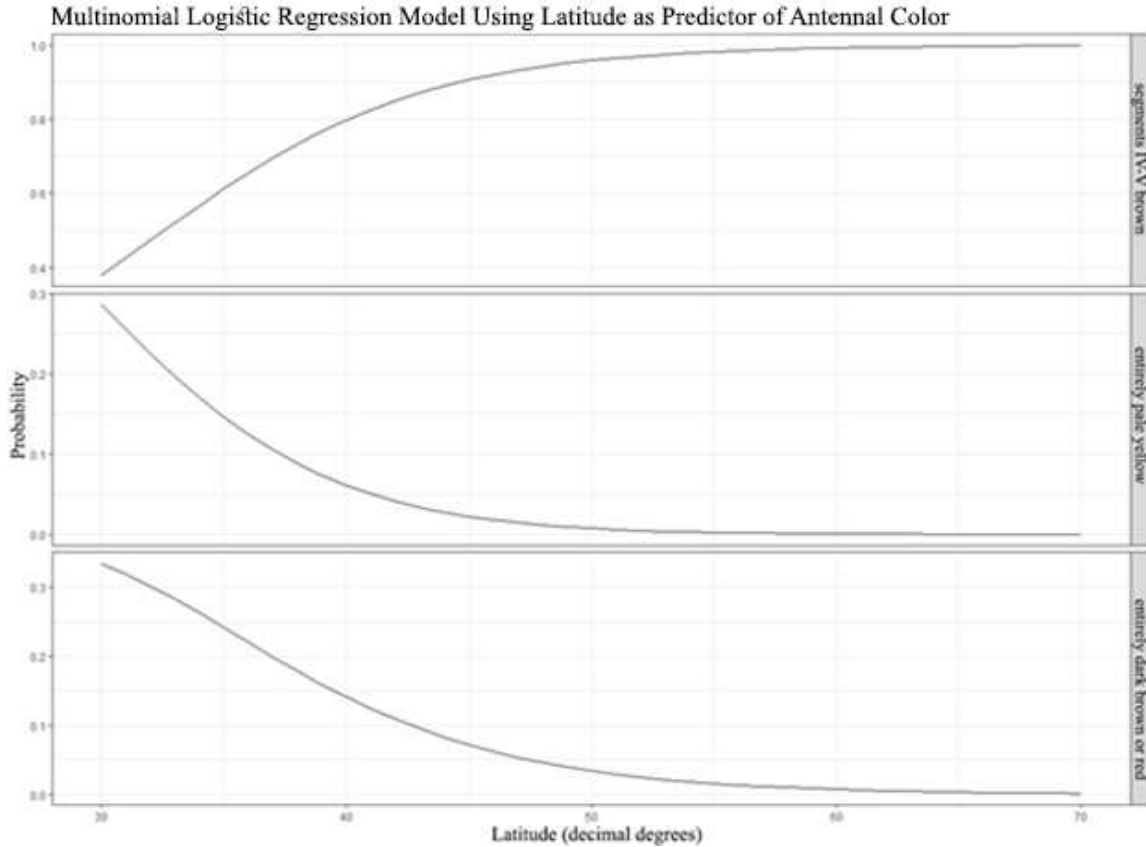


Figure 2.9. Multinomial linear regression of the predictor latitude on antennal color state demonstrating the relationship between the two variables, likelihood ratio tests validate the use of latitude as a predictor versus a model with no predictor.

2.4.2. Traditional Morphometrics: Length of Juga and Relative Length of Juga and Tylus

A one-way analysis of variance showed no significant effect of morphoform on the relative lengths of the juga [$F(2,436) = 1.408, p = .245$] (Fig. 2.10). A simple linear regression was conducted to investigate the relationship between latitude and average jugal length. The scatterplot showed that there was a linear relationship between the two variables ($p = 0.0001$) (Fig. 2.11). This data suggests that there is a linear relationship amongst average jugal length among specimens, however, there is no significant difference in this metric between morphogroups.

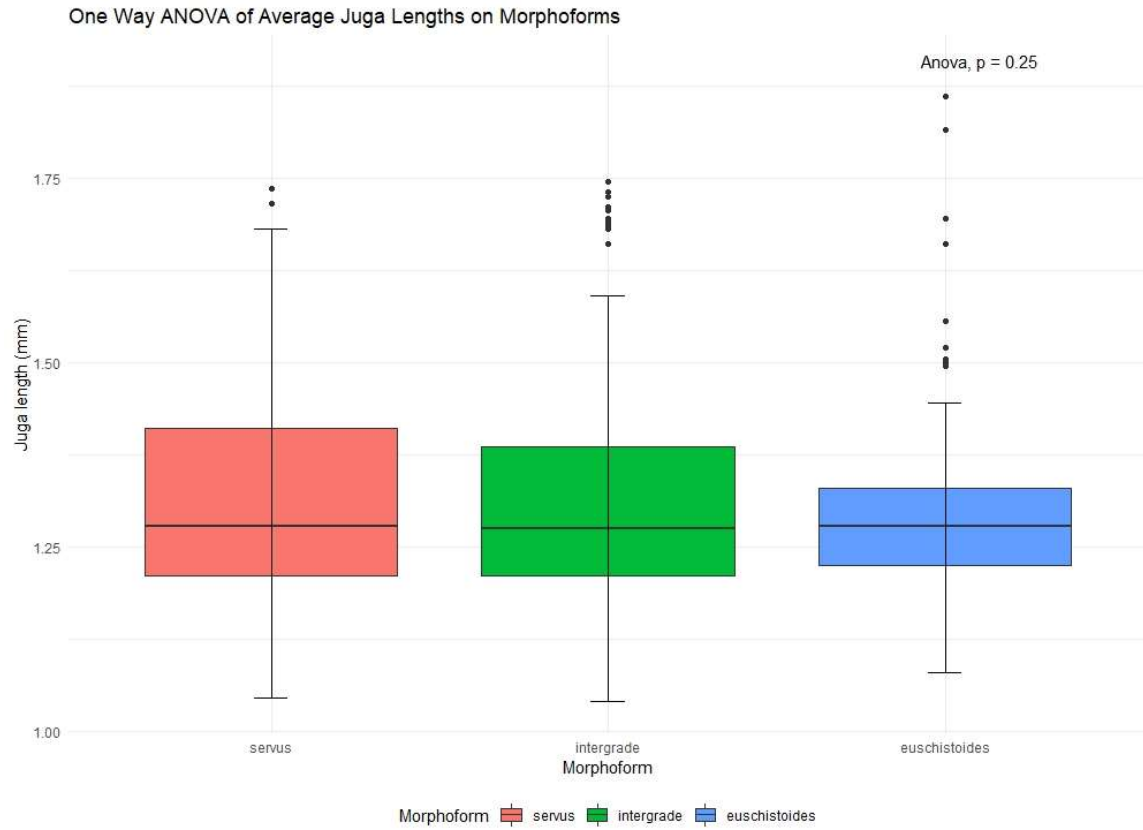


Figure 2.10. One way ANOVA determining whether average jugal length varied between morphogroups.

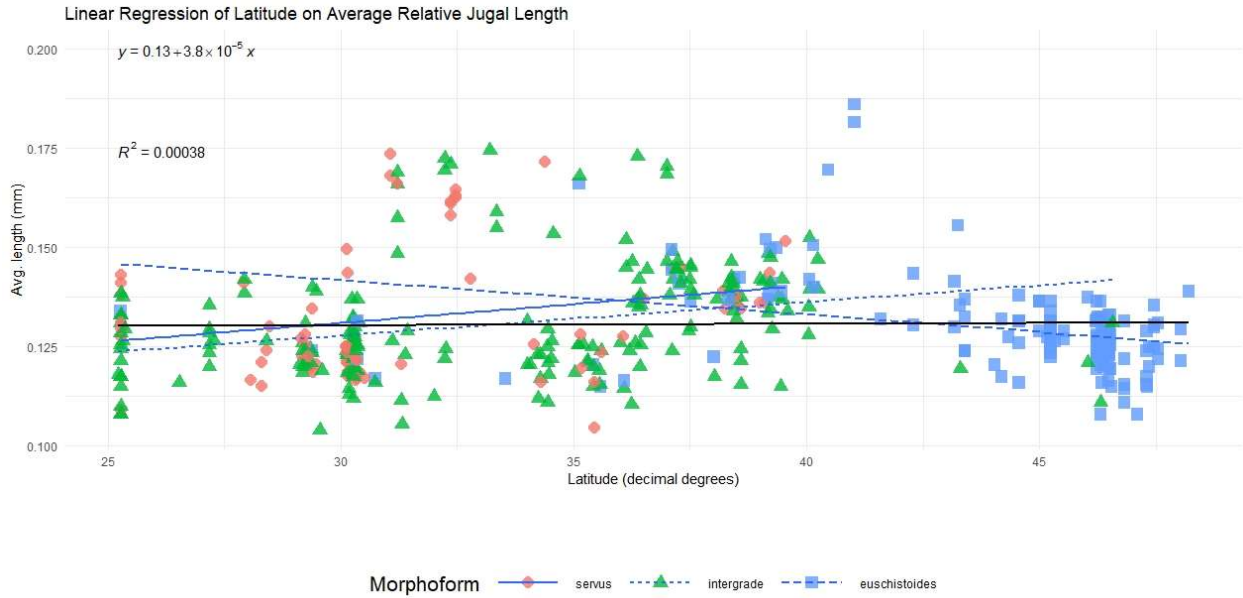


Figure 2.11. Linear regression of latitude on the average relative jugal length demonstrating that there is a significant effect ($p = 0.0001$) of latitude on the measurement with no clear directional linear relationship between the two variables.

A one-way analysis of variance showed a significant effect of morphoform on the relative length of the juga and tylus [$F(2,436) = 137.2, p = 0.0001$] (Fig. 2.12). Post hoc comparisons using the Tukey HSD test indicated that the mean relative lengths of the jugas and tylus were significantly different between all three forms. A simple linear regression was conducted to investigate the relationship between latitude and relative lengths of jugal and tylus. The scatterplot showed that there was a significant linear relationship between the two variables ($p = 0.0001$) (Fig. 2.13). This data suggests that as the latitude increases, so does the relative juga and tylus lengths which indicates that *E. servus* specimens will likely exhibit greater jugal length and more often exhibit *euschistoides*-like head features the further north specimens are located.

One way ANOVA of Relative Length of Juga and Tylus between Morphoforms

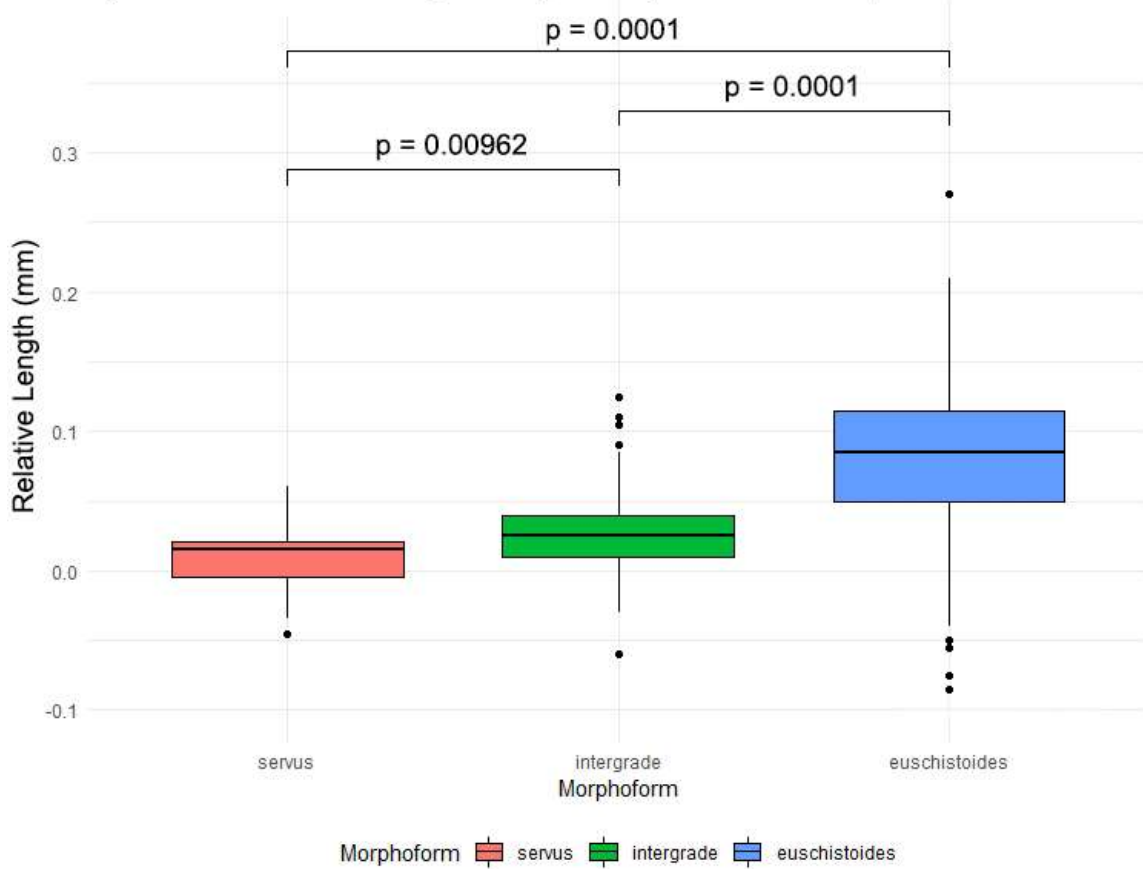


Figure 2.12. One way ANOVA determining whether relative jugal and tylus lengths varied between morphogroups.

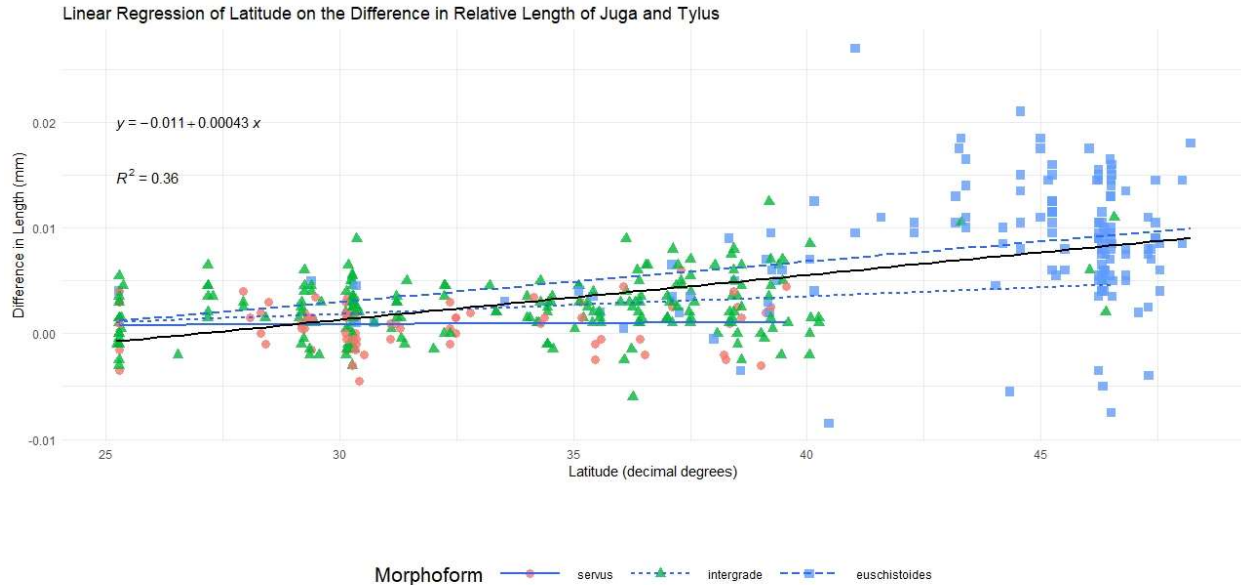


Figure 2.13. Linear regression of latitude on relative difference in lengths of juga and the tylus demonstrating there is a significant effect ($p = 0.0001$) of latitude on jugal lengths and a positive linear relationship between the two.

2.4.3. Geometric Morphometrics: Analysis of Head Shape

Centroid sizes were used as the overall measure of shape differences within the heads of specimens studied. The *euschistoides* form accounted for the highest centroid size value which suggests that it had the greatest variation from the mean shape (*i.e.*, the greatest juga to tylus ratio). The Kruskal-Wallis test found that morphoform had a relatively small effect on centroid sizes. Significant differences ($H = 0.00918$, $p = 0.011$, $df = 2$) in centroid sizes were found among the three morphoforms. The pairwise comparison shows that the intergrade forms were significantly different from *euschistoides* forms (Fig. 2.14).

Comparison of Centroid Sizes between Morphoforms

Kruskal-Wallis, $\chi^2(2) = 8.98$, $p = 0.011$, $n = 764$

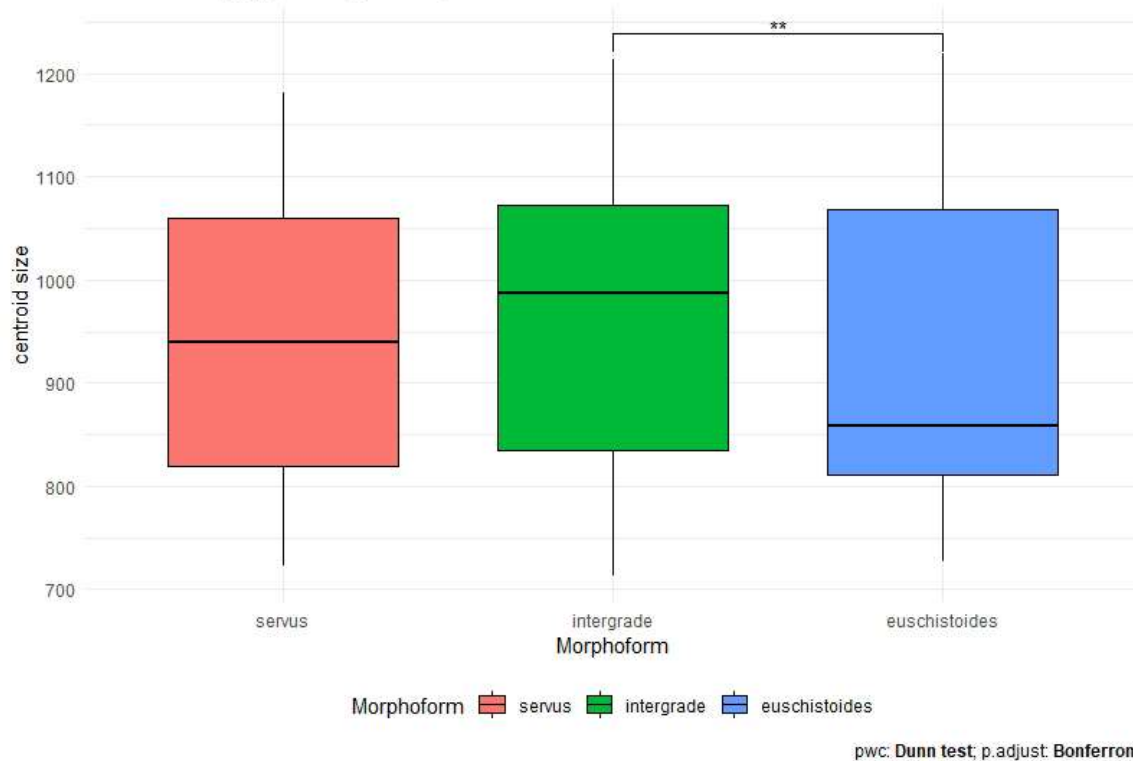


Figure 2.14. Boxplot comparing centroid sizes between *E. servus* specimen morphoforms showing significant difference between intergrade and *euschistoides* morphoforms.

A simple linear regression was conducted to investigate the relationship between latitude and centroid size. The regression demonstrated that there was a correlation between the latitude and centroid size ($p = 0.0001$), however, this model does not explain much of the variability present within the data (Fig. 2.15).

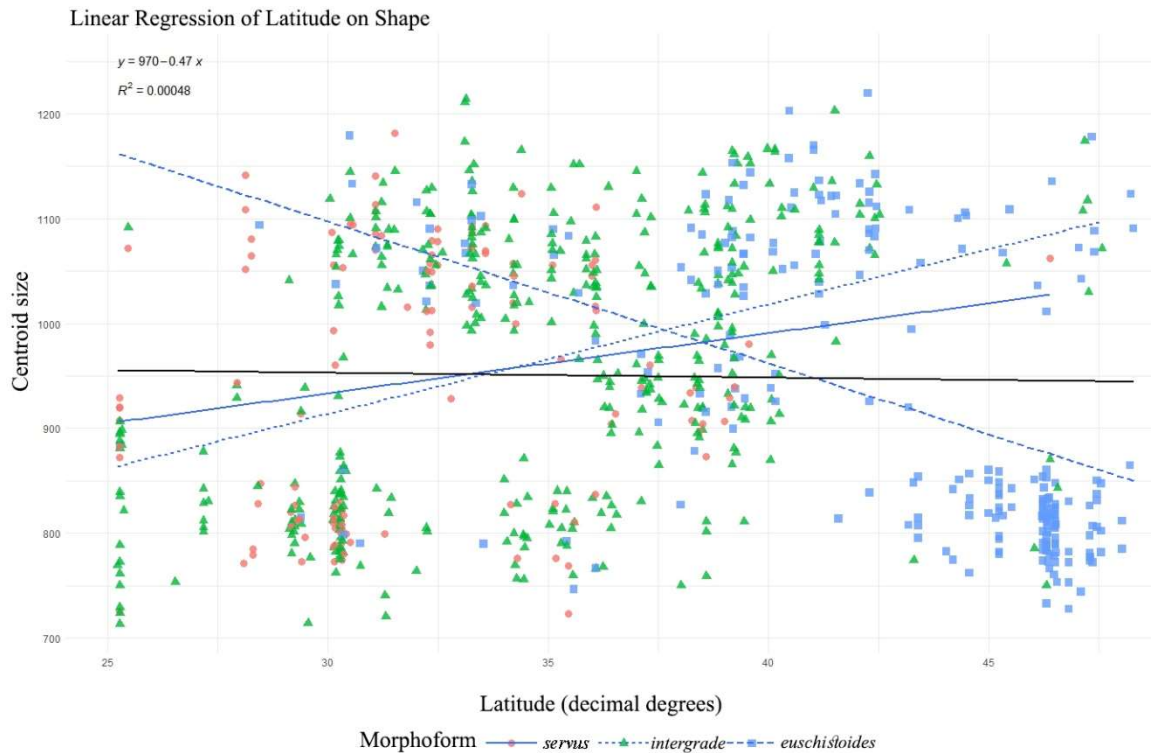


Figure 2.15. A simple linear regression on latitude and centroid size demonstrated that there was a correlation between the latitude and the centroid size ($p = 0.0001$) however, this model does not explain much of the variability.

2.4.4. Geometric Morphometrics: Principal Component Analysis

The PC analysis based upon the nine head landmarks resulted in PC1 and PC2 summarized as 52.45% of the total variance (Fig. 2.16). Landmark points 1, 2, and 9 have the greatest contribution to PC1 and PC2 (Fig. 2.17) indicating that these landmarks account for greatest amount of variance within the data. The distribution of individuals along the two PCs are shown in Fig. 2.18. The PCA displays a large data convergence in the center of the morphospace suggesting that all morphoforms were found in the center of the data (correlating with the *E. servus* intergrade zone). Further, we see that the groups overlap forming a gradient indicative of a cline. If the morphoforms were discrete taxa, the points would form distinct clusters according to their morphoforms. If they were subspecies, we would see more concrete clusters with less overlap.

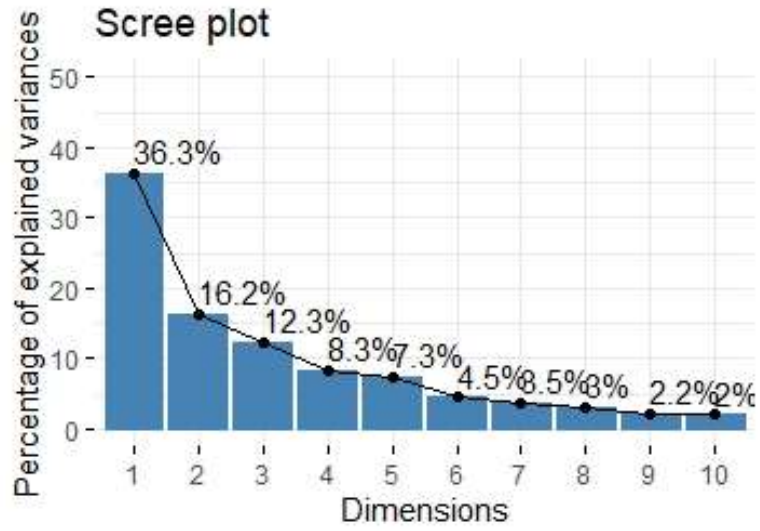


Figure 2.16. Scree plot depicting variances by principal components. PC 1 and PC 2 summarizes 52.5% of the total variance.

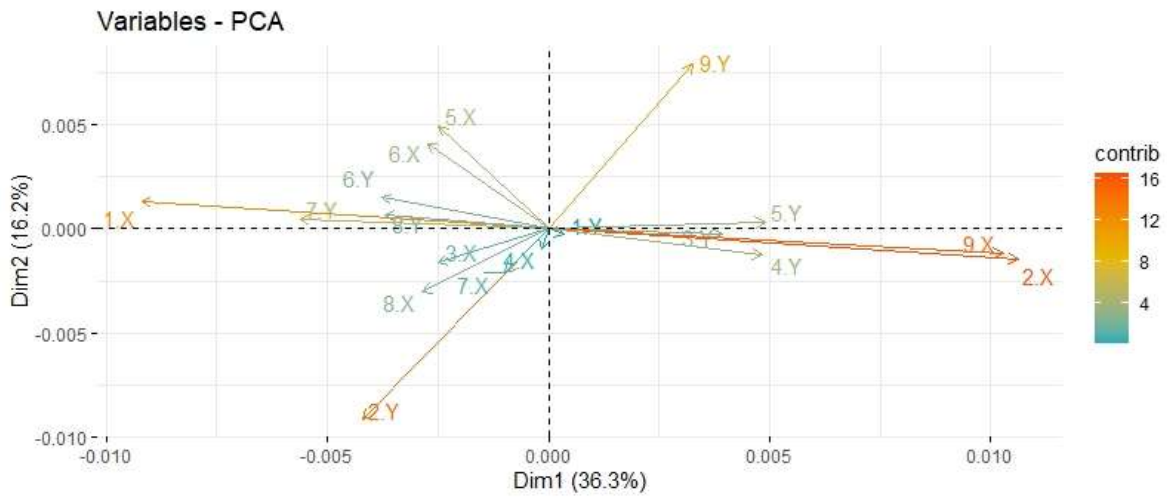


Figure 2.17. Variable contribution of each landmark coordinate to principal axes.

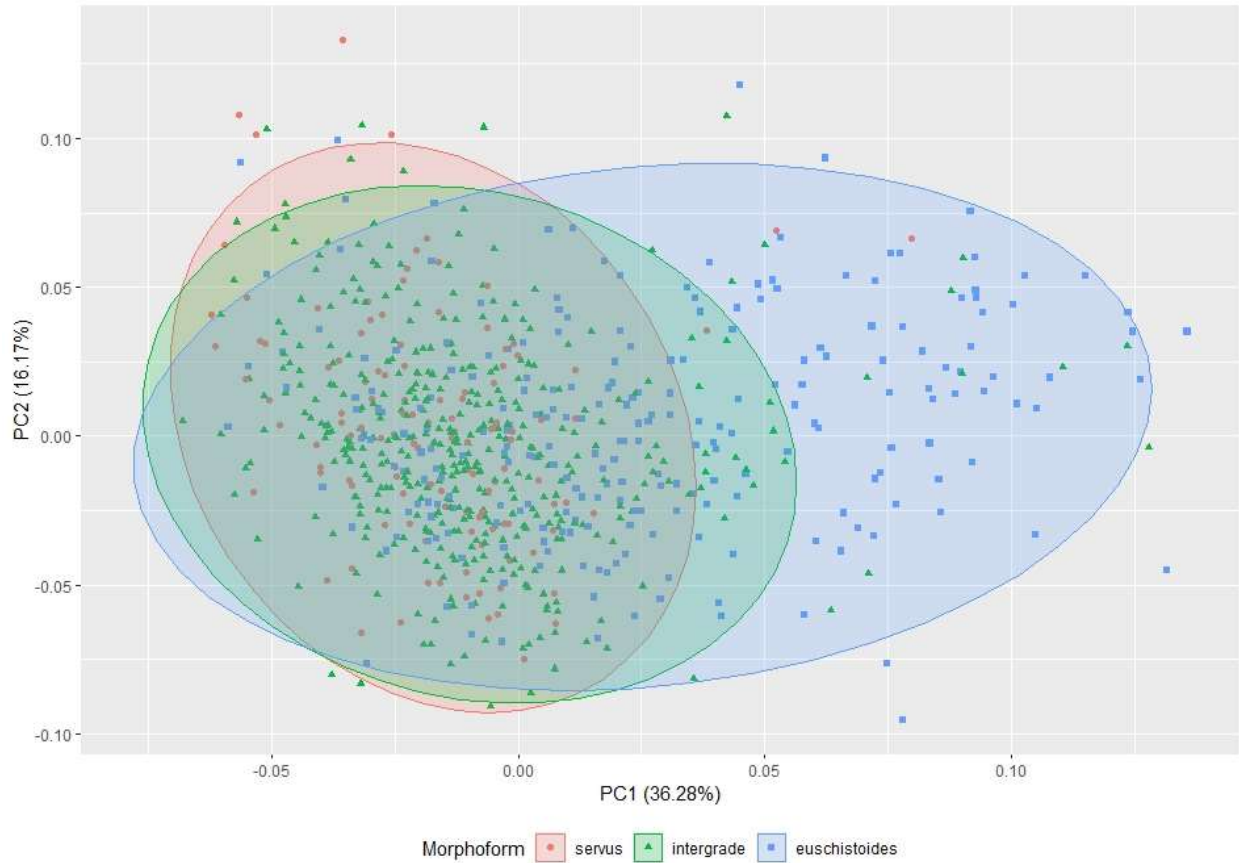


Figure 2.18. Morphospace of individual shape scores from the principal component analysis (PCA). Each dot represents a specimen grouped by morphoform.

Thin-plate splines (TPS) deformation grids were utilized to graphically depict shape changes based on landmarks along PC axes compared to the mean shape (Fig. 2.20). The shape at PC 1 max depicted individuals with a greater jуга to tylus ratio, which correlated to the individuals sampled at higher latitudes. In contrast, the shape at PC 1 min depicted individuals with the length of the jуга to tylus ratio essentially subequal, which correlated to the individuals sampled at lower latitudes. This trend repeated itself along the axis of PC2 (Fig. 2.19). The mean shape was identified as specimen 0437 specimen, which was identified as an intergrade form with a >1 jуга:tylus ratio. This specimen falls within the cluster of overlapping forms within the morphospace (Fig. 2.18). Shape changes were primarily observed in the displacement of landmark 1 (the apical point of the tylus) demonstrating the change in relative length of the jуга

and tylus. Landmarks 2 and 9 shifted slightly contributing to head shape variation within some of the specimens (Fig. 2.21).



Figure 2.19. Specimen identified as the mean shape for the data. Specimen 0437 was collected in Santa Cruz, AZ and was identified as an intergrade form.

Thin-plate Spline Deformation Grids

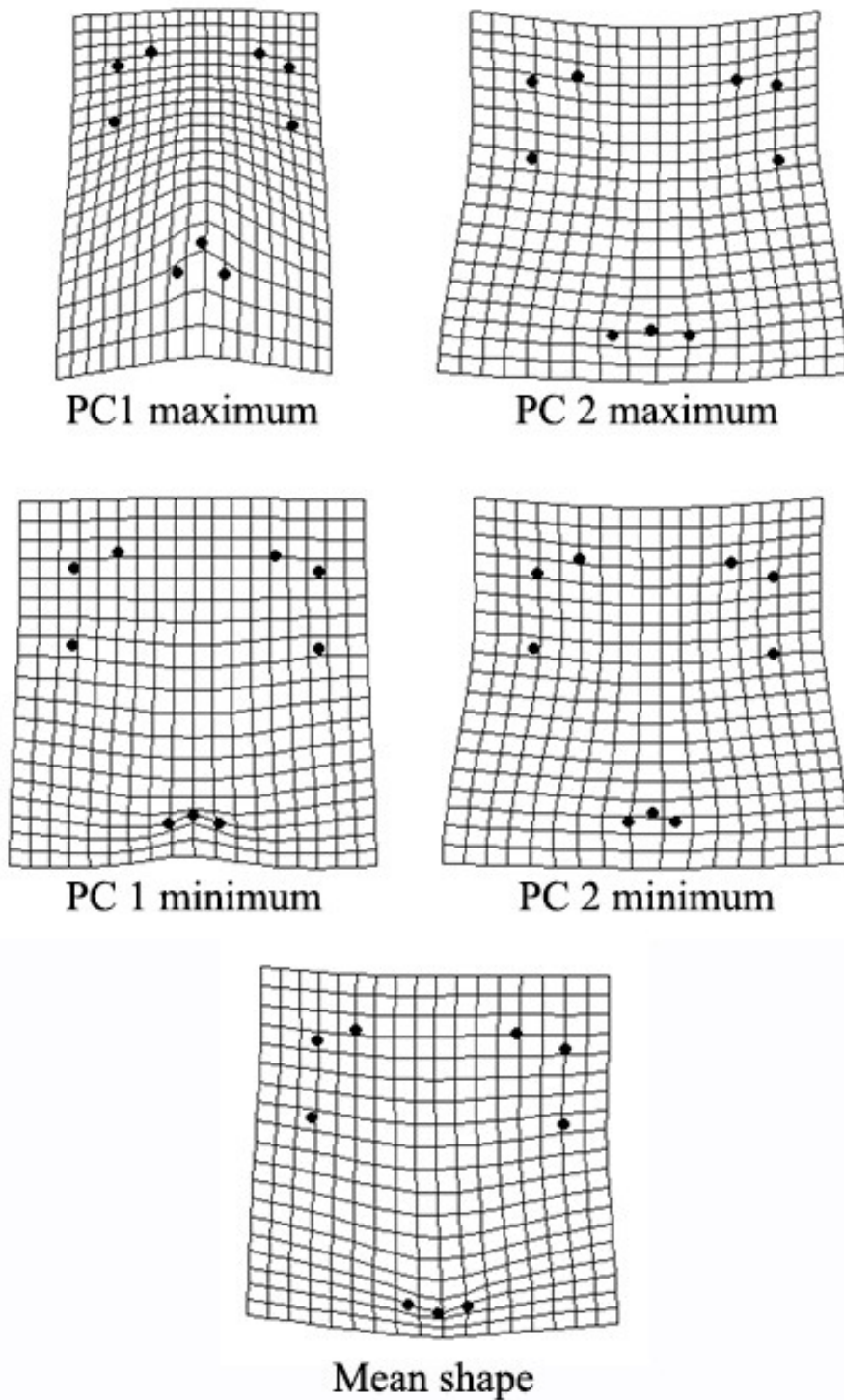


Figure 2.20. Thin-plate spline deformation grids. These grids compare specimen centroid shapes at min/max of both principal axes compared to the mean shape.

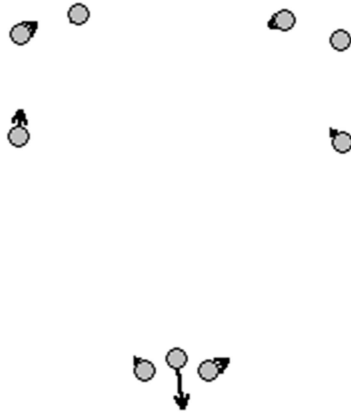


Figure 2.21. Lollipop shape change graph depicting displacement of landmark 1 demonstrating changes in the length ratio of juga and tylus.

2.5. Discussion

Both *E. s. servus* and *E. s. euschistoides* were originally described as full species. *Euschistus euschistoides* was later reduced to a subspecies (Sailer 1946). These subspecies are distinguished primarily by the variability in the relative lengths of the juga and tylus, differences in their antennal color, and whether the connexivum is exposed or not. Blatchley's (1926) suspicion of the validity of these subspecies, and McPherson's (1974) breeding experiments with *E. tristigmus* under difference environmental parameters prompted this study using a combination of morphometrics.

Utilizing a combination of traditional morphometrics with landmark-based GM we were able to investigate potential differences in the taxonomically significant morphological characteristics within the *E. servus* subspecies complex. A “good” example of a subspecies would exhibit significant differences in between characteristics (Winker 2010). However, we found no significant differences amongst average jugal lengths or the centroid sizes within

morphoforms. Our data did reveal a significant difference in the relative lengths of juga and tylus between morphoforms. The morphospace revealed a large convergence of data that is characteristic of a large intergrade zone which is not usually considered to be a defining characteristic of a ‘good’ subspecies.

Evidence from multiple analyses exhibited the positive linear relationship of latitude on each taxonomic characteristic. Multinomial logistic regression models revealed a clear relationship between latitude and the state of connexiva (exposed or not) and antennal coloration mirroring the geographic distribution of the *E. servus* subspecies. *Euschistus servus* specimens collected below 45 degrees latitude are just as likely to exhibit entirely pale yellow or entirely dark brown or red antennal coloration. We suspect that *E. servus* antennal coloration may be impacted by climate effects throughout its range. However, we are unsure of the mechanism driving the co-occurrence of *E. servus* antennal colors and connexival forms. We see an apparent inverse of Gloger’s rule in the antennal coloration; that is *E. s. euschistoides* has pale antennae with segments IV and V dark in the north, whereas *E. s. servus* has the antennae wholly pale yellow or brownish red in the south. This may be explained by the previous established notion that low temperatures and higher latitudes favors melanin development in arthropods (Dobzhansky 1933, Vernberg 1962, Rapoport 1969).

The positive linear relationships between latitude and the relative length of the juga and tylus could indicate clinal variation of the head shape. This translates in head shapes that become more *euschistus* like the further north in latitudes one travels. Principal component analysis on landmark-based GM yielded no consistent clusters with an assignment into morphoforms of *E. servus*, intergrade, or *E. euschistoides*. In addition, PCA and TPS grids displayed a clear change in headshapes along the PCs. These findings all provide evidence of supporting the notion that *E.*

servus characteristics are subject to clinal variation patterns of geographic variation. Species with broad continental distributions may be susceptible to clinal variation (gradation in one or more physical characteristics within a taxon, especially between different populations). These clines occur most commonly within continental ranges where a continuous series of populations are found (e.g., *E. servus*) (Mayr and Ashlock 1991).

The decision to choose landmark-based geometric morphometrics (GM) versus outline-based GM depends on the species chosen for any study, both significantly classify taxa. We chose landmark-based GM initially because of the relatively low demand on time in addition to the specimen requirement. Landmark-GM requires specimens that number twice the amount of landmarks (Chaiphongpachara 2018). We found that relative lengths of the juga and tylus was the primary source of variation in the GM analysis. In hindsight, we speculate if parameter length resulting from an outline analysis would have been better suited to capture the few points of shape difference amongst specimens considering that we utilized a sample size beyond that which was required ($n = 768$). While there is no clear consensus on whether one technique may claim superiority over the other, there are some authors who claimed that outline analysis performed better when discriminating close forms (Dujardin et al. 2014). However, our data collection was limited to non-destructive sampling which may have prevented from obtaining precise images of *E. servus* heads due to the fragile nature of museum specimens.

I question the value of using landmark-based geometric morphometrics for a single measurable morphometric trait when we may have achieved similar results utilizing solely traditional morphometrics. Geometric morphometrics has been criticized for the lack of methods available for assessing landmark precision and the problem introduced when defining anatomical descriptions of landmarks against precise definitions of landmarks (von Cramon-Taubadel et al.

2007). Watanabe (2018) stated that a formal treatment of whether sampled landmarks adequately shape variation is elusive but essential. He proposed a tool (LaSEC) that would “(1) identify under- and oversampling of landmarks; (2) assess robustness of morphological characterization; and (3) determine the number of landmarks that can be removed without compromising shape information.” This tool would potentially have offered this study a systematic approach to evaluating and refining the collection of datapoints. In retrospect, our landmarks may have faced several issues 1) too few characteristics assessed, 2) under-sampling, and 3) lack of evaluation of the landmark data.

Our study addresses several key points of contention regarding the subspecies concept being subject to sampling error: 1) not addressing concordant change over broad distances (Gillham 1956), 2) addressing geographic variation, and 3) use of too small of a sample size (Wilson and Brown 1953), however, we faced another set of problems. The outcome of our statistical analysis was driven by sampling size (Winker 2010) and the p-value itself is arbitrary (Halsey et al. 2015). Addressing these issues required us to incorporate a larger sample size from a greater geographic area. Both are critical to verifying initial diagnoses of subspecific designations (Winker 2010).

A well-defined subspecies may be used to discuss potential incipient species, refer to geographic subsets of species populations, note geographic variation in morphology (i.e., designate clear morphological difference or designate clear color-pattern selection), and provide a foothold to understand distribution, diversity, and microevolutionary processes (Winker 2010, Hillis 2020). We propose an integrative approach when evaluating or designating subspecific designations. In the future a combined approach should be used rather than solely focusing on morphometrics, genetics, and morphology.

2.6. Conclusion

The present study sought to reevaluate the subspecific designations within a complex of forms in the Insecta (the genus *Euschistus* in the Heteroptera: Pentatomidae) utilizing a combination of morphometric approaches. Principal component analysis assessing shape between morphogroups failed to produce clusters commensurate to the *E. s. servus* and *E. s. euschistoides* forms and when assessing the relative lengths of juga and tylus, the state of the connexiva, and antennal color, it is apparent that there is a linear relationship between latitude and these characters, more consistent with a character gradient. It is concluded that this represents an apparent clinal variation in multiple characters of the *E. servus* subspecies complex. When geographic variation within a species appears to be clinal in nature, it should not be recognized as a subspecies (Mayr 1963, Mayr and Ashlock 1991). The data supports the finding that *Euschistus servus* should be recognized as polymorphic as opposed to polytypic.

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**APPENDIX. HOST PLANTS THAT E. SERVUS HAS BEEN COLLECTED ON
THROUGHOUT ITS RANGE (ADAPTED FROM MCPHERSON 1982)**

Host Plant	Scientific name	Host Plant	Scientific name	Host Plant	Scientific name
alfalfa	<i>Medicago sativa</i>	elderberry	<i>Sambucus spp.</i>	Soybean	<i>Glycine max</i>
Apple	<i>Malus domestica</i>	elm	<i>Ulmus spp.</i>	<i>Spartina consocias</i>	<i>Spartina consocias</i>
basketflower	<i>Centaurea americana</i>	evening primrose	<i>Oenothera biennis</i>	squash	<i>Cucurbita spp.</i>
bead-grass	<i>Paspalum bushii</i>	goldenrod	<i>Solidago spp.</i>	sumac	<i>Rhus spp.</i>
bean	<i>Fabaceae spp.</i>	grape	<i>Vitis spp.</i>	sunflower	<i>Helianthus annus</i>
beech	<i>Fagus spp.</i>	hawthorn	<i>Crataegus monogyna</i>	sweet clover	<i>Melilotus officinalis</i>
beefsteak plant	<i>Perilla frutescens</i>	honeysuckle	<i>Lonicera spp.</i>	Thistle	<i>Silybum spp.</i>
birch	<i>Betula spp.</i>	horse-weed	<i>Erigeron canadensis</i>	timothy	<i>Phleum pratense</i>
black walnut	<i>Juglans nigra</i>	Indian hemp	<i>Apocynum cannabinum</i>	Tobacco	<i>Nicotiana tabacum</i>
buckhorn plantain	<i>Plantago lanceolata</i>	ironweed	<i>Vernonia spp.</i>	Tomato	<i>Solanum lycopersicum</i>
bush-clover	<i>Lespedeza thunbergii</i>	johnson grass	<i>Sorghum halepense</i>	wheat	<i>Triticum aestivum</i>
Cabbage	<i>Brassica oleracea</i>	maple	<i>Acer spp.</i>	White campion	<i>Silene latifolia</i>
camphorweed	<i>Heterotheca subaxillaris</i>	mesquite	<i>Prosopis spp.</i>	white clover	<i>Trifolium repens</i>
Canada thistle	<i>Cirsium arvense</i>	Mexican-tea	<i>Dysphania ambrosiodes</i>	white-top fleabane	<i>Erigeron vernus</i>
canadian wild rye, common	<i>Elymus canadensis</i>	Mullein	<i>Verbascum spp.</i>	wide-leaved spiderwort	<i>Tradescantia virginiana</i>
cantaloupe	<i>Cucumis melo</i>	oak	<i>Quercus spp.</i>	wild carrot	<i>Daucus carota</i>
<i>Carex socias</i>	<i>Carex socias</i>	oats	<i>Avena sativa</i>	Wild Hydrangea	<i>Hydrangea arborescens</i>
Carolina cranesbill	<i>Geranium carolinianum</i>	okra	<i>Abelmoschus esculentus</i>	wild yam	<i>Discorea villosa</i>
catnip	<i>Nepeta cataria</i>	orchard grass	<i>Dactylis glomerata</i>	yarrow	<i>Achillea millefolium</i>
cherry	<i>Prunus avium</i>	Ox-eye daisy	<i>Lecantheum vulgare</i>	yellow thistle	<i>Centaurea americana</i>
chicory	<i>Cichorium intybus</i>	panic-grass	<i>Panicum spp.</i>		
Citrus	<i>Citrus spp.</i>	Peach	<i>Prunus persica</i>		
clover	<i>Trifolium spp.</i>	pecan	<i>Carya illinoensis</i>		
cocklebur	<i>Xanthium spp.</i>	pepper	<i>Capsicum annuum</i>		
common plantain	<i>Plantago major</i>	pigweed	<i>Amaranthum spp.</i>		
corn	<i>Zea mays</i>	poplar	<i>Populus spp.</i>		
cotton	<i>Gossypium spp.</i>	prickly lettuce	<i>Lactuca serriola</i>		
crimson clover	<i>Trifolium incarnatum</i>	purple-flowered thistle	<i>Cirsium edule</i>		
crownvetch	<i>Securigera varia</i>	Ragweed	<i>Ambrosia spp.</i>		
curly dock	<i>Rumex crispus</i>	raspberry	<i>Rubus spp.</i>		
daisy fleabane	<i>Erigeron strigosus</i>	red clover	<i>Trifolium pratense</i>		
Tick-trefoil	<i>Desmodium spp.</i>	reed canary grass	<i>Phalaris arundinacea</i>		
Dogwood	<i>Cornus spp.</i>	Coneflowers	<i>Rudbeckia spp.</i>		