MOLECULAR PHYLOGENY AND REVISION OF SPECIES GROUPS OF NEARCTIC BOMBARDIER BEETLES (Carabidae: Brachininae: *Brachinus*).

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by Raine Ikagawa, titled *Rediscovering Neobrachinus Erwin: A Molecular Approach to Investigate the Evolution of Morphology and Examine Biogeographical Patterns* and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.

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Abstract

Bombardier beetles of the genus *Brachinus* Weber are notorious for their explosive defensive chemistry; when threatened, they aim a 100°C cloud of benzoquinones directly at an enemy. Despite ongoing research on their defensive chemistry and ecology, the group lacks a robust molecular phylogeny. In this study, three loci from both the mitochondrial and nuclear genome (COI, CAD, 28S) were used to reconstruct the phylogeny of Nearctic *Brachinus* and to test hypothesized relationships based on a previous study based upon morphological characters (Erwin, 1970). All molecular phylogenetic analyses recovered all species groups proposed by Erwin as monophyletic except for the *fumans* species group. We find that Erwin's *fumans* species group is polyphyletic. In its place, we erect the following five species groups: *cinctipennis*, *fumans* (revised), *galactoderus*, *phaeocerus*, and *quadripennis*. External morphological characters were traced on the molecular phylogeny to identify potential diagnostic characters. Apomorphic characters were identified for two new species groups, the *fumans* (revised) and *phaeocerus* groups. We also expand the *cordicollis* group to include *B. medius* and the *americanus* group.

Chapter 1: Introduction

Bombardier beetles of the genus *Brachinus* Weber are famous for their explosive defensive chemistry; when provoked, they can generate a 100°C cloud of benzoquinones and aim the explosion towards the threat (Aneshansley et al. 1969; Dean et al. 1990; Eisner et al. 1958; Schildknecht et al. 1964). *Brachinus* are abundant scavengers in their communities, they offer other carabids (including *Agonum*, *Platynus*, and *Chlaenius*) with well-protected spaces within their large diurnal multispecies aggregations (Schaller et al. 2018) and have the potential to sustainably manage pest populations in agroecosystems (Scaccini et al. 2020). Although *Brachinus* are most well-known for their defensive chemistry, previous research has also examined other interesting aspects of their life history, behavior, and ecology; including larval development as ectoparasites of beetle pupae (Wickham 1894; Erwin 1967; Juliano 1984; Juliano 1985, Saska and Honek 2004), multispecies aggregation behaviors (Pérez Zaballos 1985; Bonacci et al. 2004; Brandmayr et al. 2006; Bonacci et al. 2008; Schaller et al. 2018), and their microbiome (McManus et al. 2018).

While research on *Brachinus* continues to be published, including numerous new species descriptions (e.g., Kirschenhofer 2010; Tian and Deuve 2015), only one study has attempted to reconstruct the phylogeny of species within the genus (Erwin 1970) . Bombardier beetles within *Neobrachinus* have historically been described as taxonomically difficult. Indeed, Ball (1960) wrote: "The taxonomy of the North American species of this group is very poorly understood and it is almost a waste of time at present to attempt to determine individuals to species." North American *Brachinus* species are notoriously difficult to identify due to highly conserved morphology and aposematic coloration; the maintenance of "the *Brachinus* habitus" seems to have been favored over the course of multiple speciation events (e.g. Fig. 1c, d, f, g, i) (Schaller et al. 2018).

Erwin's (1970) immense effort to remedy this situation required careful examination of over 28,000 specimens of *Brachinus* and over 2,000 specimens of other brachinine taxa. Using morphology, he determined that all New World *Brachinus* belong to a single monophyletic group, *Neobrachinus* Erwin, 1970. He used morphological characters to classify 62 members of *Neobrachinus* into 14 species groups (representatives in Fig. 1) and used those same characters to propose a species group tree (Fig. 2).

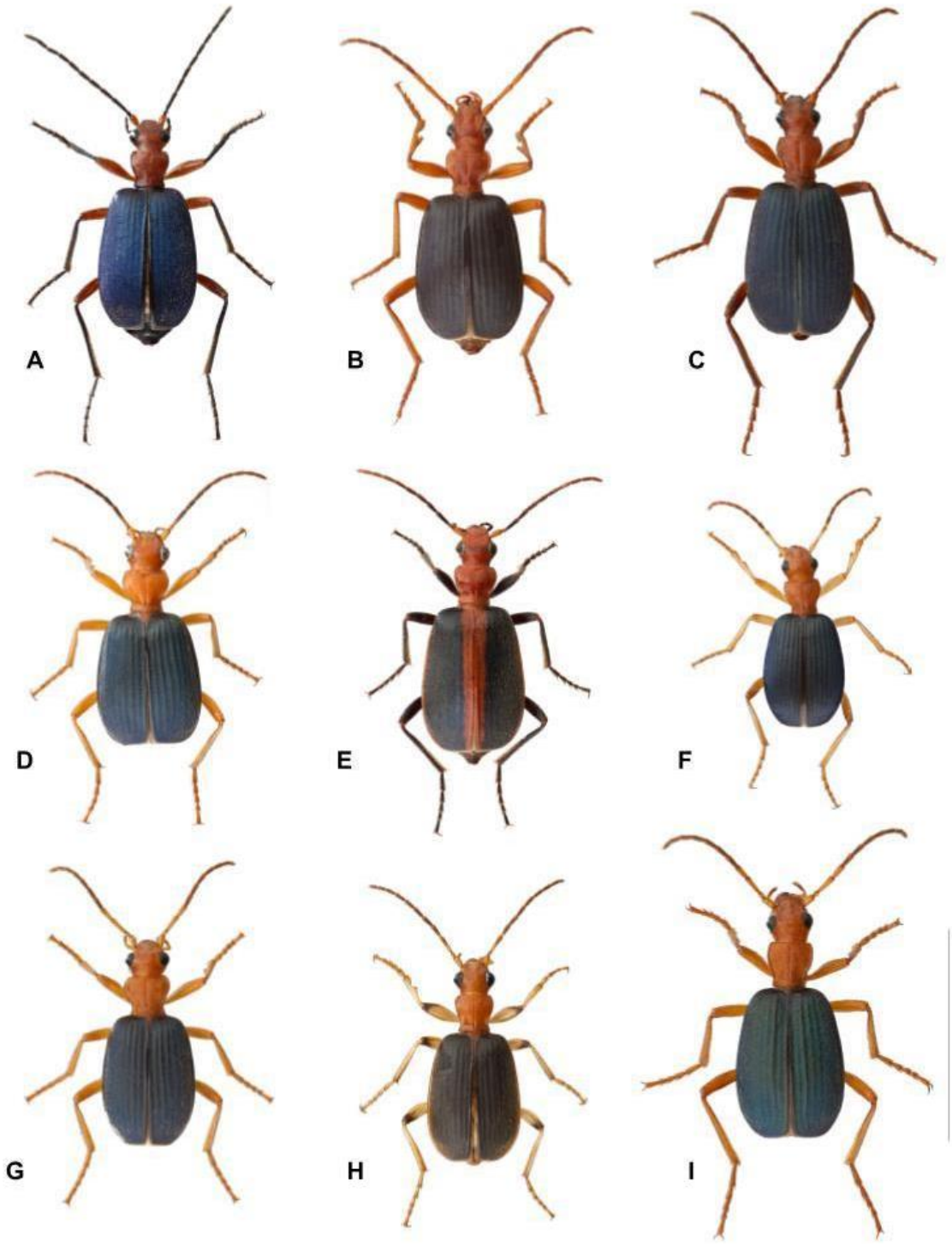

Figure 1. Dorsal habitus view of representatives from several species groups of *Neobrachinus*. Scale bar = 1cm. **A.** *B. azureipennis*. **B.** *B. gebhardis*. **C.** *B. elongatulus*. **D.** *B. mexicanus*. **E.** *B. cibolensis*. **F.** *B. costipennis*. **G.** *B. hirsutus*. **H.** *B. lateralis*. **I.** *B. favicollis*. Photos by Chip Hedgcock.

Erwin (1970) relied heavily on the shape of the virga (the apical sclerite surrounding the gonopore of the male endophallus), to define species groups within *Neobrachinus*. The *americanus* group was placed at the base of the tree, based on the similarities in the shape of the virga with *B. dryas* Andrewes, a species outside of *Neobrachinus*, known from Sikkim, India (Erwin, 1970). Erwin regarded the virga of *B. dryas* as a *Neobrachinus*-type, different from all other virgae of *Brachinus* species outside of the Americas (Erwin, 1970). In this scenario, an ancestral *Brachinus*, which also gave rise to *B. dryas* in Asia, crossed the Bering Land Bridge into North America and radiated into the 62 species of *Neobrachinus* seen today (Erwin, 1970). Other characters, including infuscation patterns, color of the elytra, and setation patterns, were also considered diagnostic for some species groups and species (Erwin, 1970).

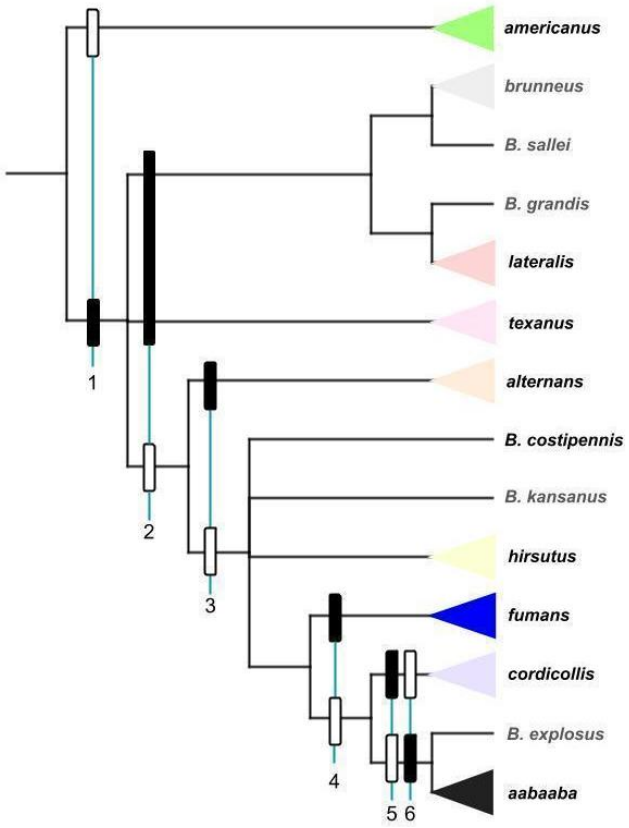

Figure 2. Species group phylogeny proposed in Erwin (1970). Species groups with representatives examined in this study have black text, while those not included are in grey font. Species groups that were proposed to contain only a single species are denoted by the species name. Apomorphic states (AS) represented by black rectangles, and plesiomorphic states (PS) represented by white rectangles, proposed by Erwin are mapped onto the phylogeny: 1. PS = pouch-shaped virga, AS = virga variously modified; 2. PS = endemic to North America, AS = endemic to South America; 3. PS = virga rounded apically, AS = virga acute apically and tripartite; 4. PS = virga unmodified, AS = virga with central ventral trough; 5. PS = virga unmodified, AS = virga "H" shaped; 6. PS = virga continuous with axis of endophallus, AS = virga perpendicular to axis of endophallus. Adapted from Erwin (1970).

Here, we use DNA sequence data to infer a phylogeny and test the relationships and boundaries of species groups of *Neobrachinus*. With challenging taxa, molecular sequence data

is often used to provide clarity, uncover diversity, and identify synonyms that morphology cannot. The subgenus *Neobrachinus* is an excellent system for using molecular data to test morphological hypotheses of phylogeny because it has been proven to be monophyletic using both morphological and molecular data (Erwin 1970, Schaller et al. 2018), many of its members are challenging to identify morphologically, and previous research has suggested that morphological characters alone are not sufficient to elucidate their phylogeny (Schaller et al. 2018). This study aims to address these challenges by using DNA sequence data to infer a phylogeny and test the relationships and boundaries of species groups of *Neobrachinus*, thus providing the evolutionary foundation for future investigations of their ecology and behavior.

Chapter 2: Methods

Taxon sampling and classification

Voucher specimens were utilized from the following institutions and individuals: University of Arizona Insect Collection (Tucson, Arizona, USA), University of Alberta E.H. Strickland Entomological Museum (Edmonton, Alberta, CA), and Evan Waite at Arizona State University (Table S1).

DNA extraction and quantification

Total genomic DNA was extracted from the right middle leg of specimens using the Qiagen® DNeasy Blood & Tissue Kit (Valencia, CA) following the manufacturer suggested protocol. Extractions on older specimens were conducted in the Schlinger Ancient DNA Laboratory at the University of Arizona Insect Collection using the QIAamp DNA Micro Kit (Qiagen Inc., Valencia, CA) following the manufacturer suggested protocol. The concentration of total genomic DNA in extraction products was measured on a Qubit 3.0 Fluorometer (Thermo Fisher, USA). Samples with quantifiable DNA were used in subsequent PCRs.

Polymerase Chain Reaction

Two genes were targeted for PCR amplification. The barcoding region, mitochondrial gene cytochrome oxidase subunit 1 (COI), was targeted using primers LCO1480 and HCO2198 (Folmer et al. 1994). The ribosomal gene 28S was targeted using primers LS58F and LS998R (Ober, 2002). Total genomic DNA that was extracted for another study (Schaller et al. 2018) was utilized and targeted for PCR amplification of the ribosomal gene 28S.

Sequencing

PCR products were quantified, normalized, and sequenced in forward and reverse directions using Sanger sequencing methods at the University of Arizona Genetics Core (UAGC) using an Applied Biosystems 3730 DNA Analyzer (ThermoFisher Scientific). Chromatograms were assembled into contigs, and initial base calls were made using Phred (Green and Ewing, 2002) & Phrap (Green 1999) as implemented by Chromaseq 1.52 (Maddison and Maddison, 2020a)

within Mesquite 3.61 (Maddison and Maddison, 2019). Final base calls were made through visual inspection of the contigs. All sequences were submitted to BOLD and GenBank (Table S1).

Sequence alignment

Three single gene matrices (COI, CAD, and 28S) were assembled with the sequences generated specifically for this study, as well as all homologous sequences of *Neobrachinus* publicly available on BOLD and GenBank (databases searched January 2021) (Supplemental Table 1). The COI matrix contained 270 taxa, the CAD matrix contained 70 taxa, and the 28S matrix contained 54. All single gene matrices were aligned using default settings in MAFFT v7.474 (Katoh and Standley 2013) within Mesquite. Genes recovered from the same specimen were concatenated in a single matrix containing 282 taxa, which comprised 228 *Neobrachinus* (representing 9/15 *Neobrachinus* species groups, and 32/62 *Neobrachinus* species) and 54 outgroup species.

Phylogenetic analysis

In the concatenated matrix and single gene matrices, COI and CAD characters were partitioned by codon position. Maximum likelihood analyses and bootstrap analyses were conducted on all four datasets using IQ-TREE version 1.6.10 (Nguyen et al. 2015). The ModelFinder feature within IQ-TREE (Kalyaanamoorthy et al. 2017) was used to find the optimal character evolution models. The ModelFinder Plus model option was used for 28S, and the TESTMERGE option was used for the protein-coding genes and for the concatenated dataset. One hundred searches were conducted for the maximum-likelihood tree for each matrix. Bootstrap analyses for the four trees were conducted with 1000 replicates using IQ-TREE version 1.6.10 (Nguyen et al. 2015), as orchestrated by the CIPRES Science Gateway (Miller et al. 2010).

Morphological character analysis

Forty-six external morphological characters that were considered diagnostic of *Neobrachinus* species and species groups in Erwin (1970) were coded for all species in the molecular phylogeny based on visual inspection of the DNA voucher specimens and detailed descriptions from Erwin (1970) (Supplemental Table 2, Supplemental Table 3). Character states were traced on the molecular phylogeny to identify potential apomorphic characters.

Chapter 3: Results

Models and partitions

IQ-Tree ModelFinder and ModelFinder Plus identified the following models of evolution for each character partition: COI codon 1 = K2P+I+G4; COI codon position 2, CAD codon 1, CAD

codon 2 = 2K2P+I; COI codon 3 = TIM2+F+G4; CAD codon 3 = HKY+F; and 28S = GTR+F+I+G4.

Molecular phylogeny

The three-gene IQ-Tree analysis resulted in the phylogeny shown in Figure 3. The full concatenated tree, and individual gene trees for 28S, CAD, and COI, are shown in Supplementary Materials 1 (Figs S1.1-1.4). The COI analysis identified several specimens that could be misidentified (Fig. S1.4). In all analyses, the following species groups with multiple representatives in this study were recovered as monophyletic: *cordicollis*, *hirsutus*, *lateralis*, and *texanus*.

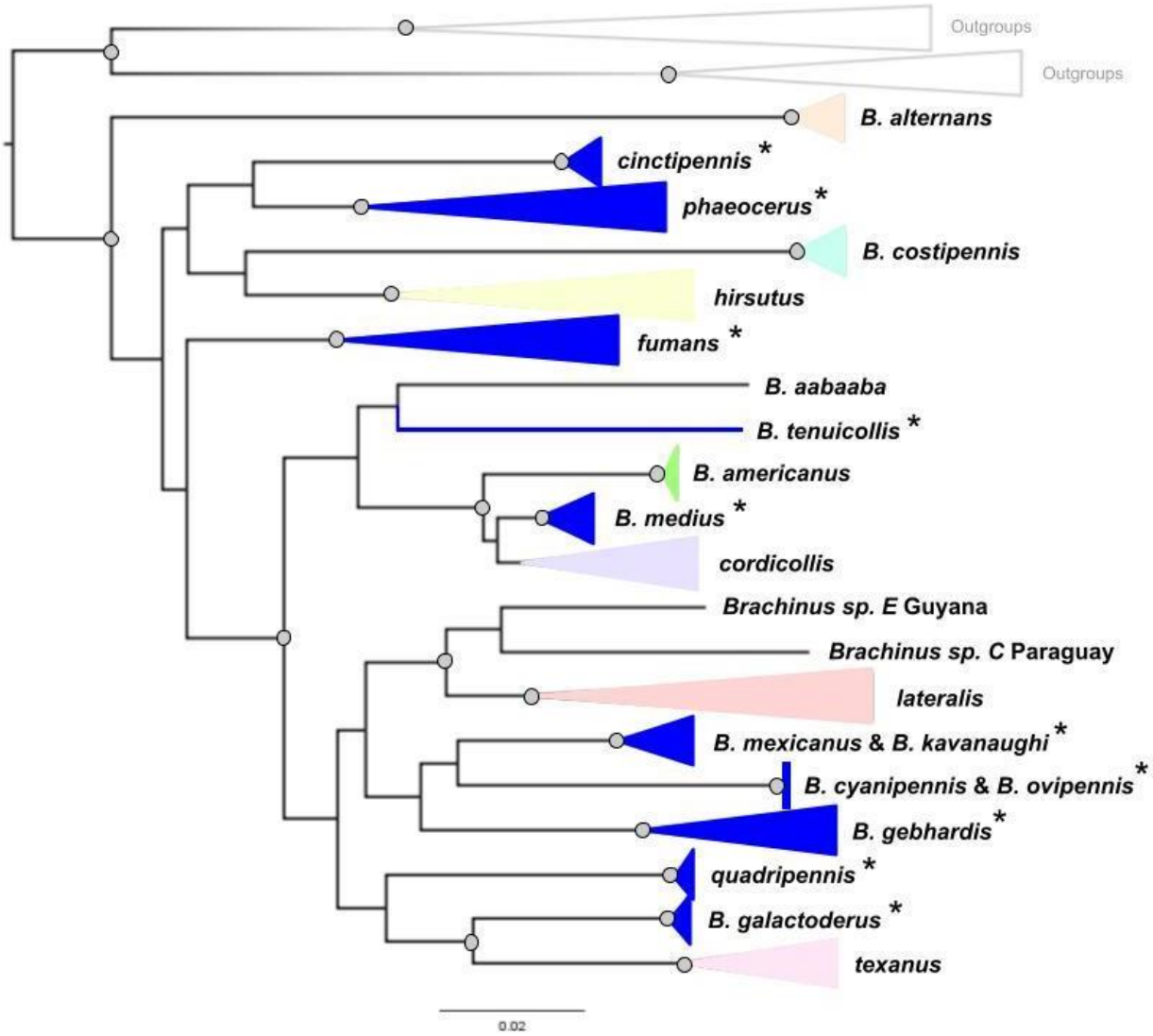

Figure 3. Maximum-likelihood three-gene molecular phylogeny of *Neobrachinus*. Clades are colored by Erwin's 1970 species group designation (compare with Figure 2). Clades with asterisks (*) were formerly placed within the *fumans* species group. Nodes with bootstrap values >90 are denoted with grey circles.

All analyses recovered the *fumans* species group proposed by Erwin (1970) as polyphyletic (Figs 3, S1.1, S1.2, S1.3, S1.4). The *quadripennis* species subgroup was split into several different clades, as was the *gebhardis* subgroup (Figs S1.1, S1.2, S1.3, S1.4). The members of the *fumans* species subgroup formed a monophyletic group (Figs 3, S1.1, S1.2, S1.3, S1.4). *B. medius* fell within a highly supported clade containing Erwin's *americanus* and *cordicollis* groups (Fig. 3, Table 1); the support for this clade warrants the expansion of the *cordicollis* group to include *B. americanus* and *B. medius*. *B. tenuicollis* was represented by a single COI sequence, and its placement remains uncertain. Bootstrap support values for each species group in each analysis are shown in Table 1.

Table 1. Bootstrap support values for the subgenus *Neobrachinus* and its species groups from each analysis: on the concatenated dataset, and individual matrices of 28S, CAD, and COI. New and revised species groups indicated with a triangle (△).

Group	Concatenated	28S	CAD	COI
Neobrachinus	99	83	98	100
cinctipennis △	100	-	-	100
cordicollis △	100	98	94	99
costipennis	100	-	100	100
fumans △	100	96	99	95
hirsutus	100	-	91	99
galactoderus △	100	-	-	100
galactoderus + *texanus* group	90	-	-	94
lateralis	90	65	70	89
lateralis + South American taxa	97	-	82	95
phaeocerus △	98	100	82	89
texanus	100	97	100	100
quadripennis	100	100	100	99

Morphological character analysis

Morphological character tracing on the molecular phylogeny identified two new apomorphic characters of new species groups of *Neobrachinus*. The *fumans* species group can be diagnosed by the rugose macrosculpture of the head extending beyond the frontal furrows behind the eyes (Fig. 4). The *phaeocerus* species group can be diagnosed by the presence of a black sutural stripe on the elytra (Figure 1a).

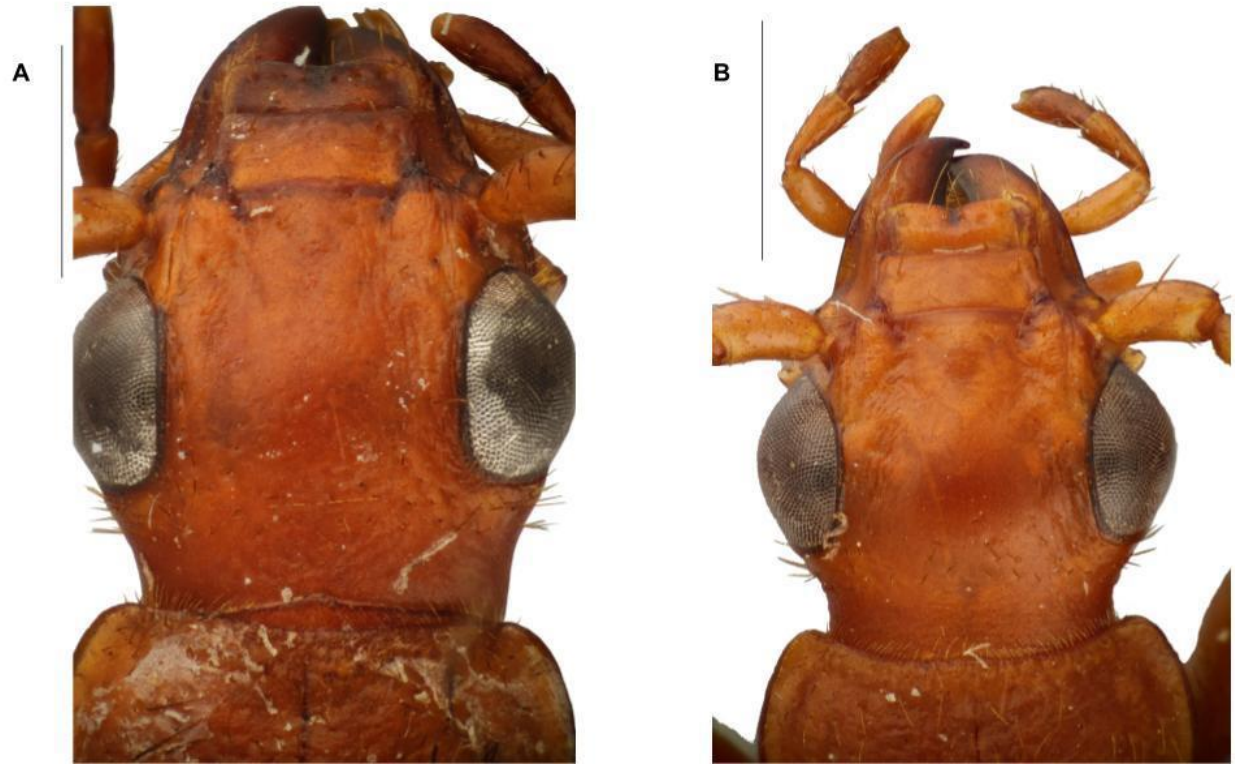

Figure 4. New diagnostic character of the *fumans* species group, and alternative character state. A. *B. fumans*. Note the extensive rugose (wrinkled) texture of the frontal furrows and behind the eyes. B. *B. mexicanus*, showing the alternative state.

Table 2: Revised classification of Nearctic *Brachinus*. New and revised species groups indicated with a triangle (△). Species groups and species not present in molecular phylogeny indicated with an asterisk (*). *Incertae sedis* taxa not considered in Erwin (1970) indicated with a circle (○).

***alternans* species group**
- *B. alternans* Dejean
- *B. rugipennis* Dejean*
- *B. viridipennis* Dejean*

***cinctipennis* species group△**
- *B. cinctipennis* Chevrolat
- *B. cibolensis* Erwin

***cordicollis* species group (revised)△**
- *B. cordicollis* Dejean
- *B. americanus* (LeConte)
- *B. alexiguus* Erwin*
- *B. capnicus* Erwin*
- *B. cyanochroaticus* Erwin
- *B. fulminatus* Erwin
- *B. ichabodopsis* Erwin*
- *B. janthinipennis* (Dejean)
- *B. medius* Harris
- *B. microamericanus* Erwin*
- *B. mobilis* Erwin
- *B. oxygonus* Chaudoir*
- *B. sublaevis* Chaudoir
- *B. vulcanoides* Erwin*

***costipennis* species group**
- *B. costipennis* Motschulsky

***fumans* species group△**
- *B. fumans* (Fabricius)
- *B. favicollis* Erwin
- *B. imperialensis* Erwin
- *B. perplexus* Dejean
- *B. puberulus* Chaudoir*
- *B. velutinus* Erwin*

***galactoderus* species group△**
- *B. galactoderus* Erwin

***hirsutus* species group**
- *B. hirsutus* Bates
- *B. pallidus* Erwin

***lateralis* species group**
- *B. lateralis* Dejean
- *B. adustipennis* Erwin
- *B. aeger* Chaudoir
- *B. arboreus* Chevrolat*
- *B. chalchihuitlicue* Erwin*
- *B. chirriador* Erwin*

***phaeocerus* species group△**
- *B. phaeocerus* Chaudoir
- *B. azureipennis* Chaudoir
- *B. consanguineus* Chaudoir*
- *B. imporcitis* Erwin
- *B. javalinopsis* Erwin

***quadripennis* species group**
- *B. quadripennis* Dejean
- *B. neglectus* LeConte

***texanus* species group**
- *B. texanus* Chaudoir*
- *B. elongatulus* Chaudoir
- *B. geniculatus* Chaudoir

incertae sedis
B. aabaaba Erwin
B. atramentarius Mannerheim○
B. bilineatus Laporte○
B. brunneus Castelnau*
B. bruchi Liebke○
B. conformis Dejean*
B. cyanipennis Say
B. explosus Erwin*
B. fulvipennis Chaudoir○
B. fuscicornis Dejean○
B. gebhardis Erwin
B. genicularis Mannerheim○
B. grandis Brullé*
B. hylaenus Reichardt○
B. immarginatus Brullé○
B. intermedius Brullé○
B. kansanus LeConte*
B. kavanaughi Erwin
B. limbiger Chaudoir○
B. marginellus Dejean○
B. marginiventris Brullé○
B. melanarthrus Chaudoir*
B. mexicanus Dejean
B. niger Chaudoir○
B. nigricans Chaudoir○
B. nigripes Waterhouse○
B. oaxacensis Erwin*
B. olidus Reiche○
B. ovipennis LeConte
B. pachygaster Perty○
B. pallipes Dejean○
B. patruelis LeConte*
B. sallei Chaudoir*
B. sonorous Erwin*
B. tenuicollis LeConte
B. vicinus Dejean○
B. xanthophryus Chaudoir○
B. xanthopleurus Chaudoir○

Chapter 4: Discussion

Neobrachinus species groups

This study used molecular data to test a previous hypothesis of *Neobrachinus* phylogeny based on morphological characters. All species groups, including those newly erected, had high support (Table 1); however the relationships between species groups, for the most part, remain unclear (Figure 3).

For example, in the concatenated analysis, the *phaeocerus* group was sister to the *cinctipennis* group, and the *fumans* group sister to another clade of *Neobrachinus* species. However, COI analysis placed the *fumans* and *phaeocerus* groups as sister groups, and the *cinctipennis* group as sister to that clade, with low bootstrap values (<55) (Fig. 3). The CAD analysis recovered the relationship between the *fumans* and *phaeocerus* groups to other *Neobrachinus* as polytomous, while in the 28S analysis the *phaeocerus* group is sister to the *hirsutus* group and *fumans* as sister to the rest of *Neobrachinus*. Each of these groups possess clear morphological apomorphies and, like all species groups recovered in this study, strong molecular support that warrant their status as separate species groups.

Some of Erwin's species groups in this study were represented by a single member, such as the *alternans* group (Table S1). Likewise, many species were represented by a single specimen, such as *B. aabaaba* and *B. tenuicollis*. Erwin's classification also included a number of monotypic species groups that were not included in this study, including the *kansanus*, *grandis*, and *sallei* groups (1970). In order to fully elucidate relationships between species groups and understand *Neobrachinus* phylogeny, greater sampling of each species, and broadening taxon sampling within *Neobrachinus*, are required.

Erwin (1970) hypothesized that the relationship of species groups within *Neobrachinus* was largely connected to the evolution of the shape of the virga (Fig. 2). The polyphyly of the *fumans* species group, as well as the molecular support for the new *cordicollis* group, do not support this hypothesis (Figs 2, 3). In Erwin (1970), the virga of the *americanus* group was considered plesiomorphic among *Neobrachinus,* while the "H-shaped" virga of the *cordicollis* group was considered highly derived (Fig. 2). The molecular phylogeny indicates a different evolutionary history of *Neobrachinus*, and of the virga, than envisioned by Erwin (1970).

Polyphyly of the fumans *species group*

All molecular phylogenetic analyses recovered all species groups proposed by Erwin (1970) as monophyletic except for the *fumans* species group. This group contained 26 morphologically diverse species and was defined by a troughed virga. In light of molecular evidence supporting the splitting of the *fumans* group, the troughed virga could be an ancestral or convergent form among *Neobrachinus*.

Species subgroups of the *fumans* group were also split in the molecular phylogeny, which also highlight potential convergent male genitalia characters. Members of Erwin's *quadripennis* subgroup of the *fumans* group were recovered throughout the *Neobrachinus* tree: in the *phaeocerus* species group, *quadripennis* species group (revised), and the clade denoted "*B. mexicanus* & *B. kavanaughi*" (Fig. 3). This group was characterized by a ridge on the ventral surface of the male genitalia. The two members of Erwin's *gebhardis* subgroup, *B. gebhardis* and *B. galactoderus*, were recovered in separate clades; this group was characterized by the form of the median lobe of the male genitalia.

We found strong support for the monophyly of the *fumans* species subgroup proposed by Erwin; it was the only subgroup within the fumans species group that was supported by molecular data (1970). This group shares a newly identified apomorphic character, the rugose texture of the head extending beyond the frontal furrows (Fig. 4). They also share characters identified by Erwin: the swollen median lobe of the male genitalia, a pale (ferrugineous) abdomen infuscated at the sides, coarsely punctate pronotum, and "generally similar habitus," (1970). Two members of Erwin's *fumans* subgroup, *B. puberulus* and *B. velutinus*, have not yet been examined molecularly. However, the presence of the apomorphic character of the rugosity of the head, and shared characters with the other members of this group, suggest that they are also members of the *fumans* clade.

An apomorphic morphological character was also identified for the *phaeocerus* group: a black sutural stripe on the elytra (Fig. 1a). The only member of Erwin's *phaeocerus* species subgroup that was not examined in this study, *B. consanguineus*, also possesses a sutural stripe, and we hypothesize that it is also a member of this group.

Redefined cordicollis *species group*

Molecular data presented strong evidence for the expansion of Erwin's *cordicollis* species group to include *B. americanus* and *B. medius* (Figs 3, S1.1, S1.2, S1.3, S1.4), as all analyses recovered this clade with high support (Table 1). However, relationships within the redefined *cordicollis* group require further investigation. Erwin's *cordicollis* group contained the following species included in the molecular study: *B. cordicollis*, *B. cyanochroaticus*, *B. fulminatus*, *B. janthinipennis*, and *B. sublaevis*. This group was supported in the concatenated and CAD analyses (Figs 3, S1.1, S1.3). However, the 28S analysis recovered relationships between *B. fulminatus*, *B. americanus*, and the rest of the *cordicollis* group as polytomous, although no 28S sequences of *B. medius* were available (Fig. S1.2). The COI analysis recovered *B. fulminatus* and *B. medius* as sister taxa, and *B. americanus* as sister to the rest of the *cordicollis* group (Fig. S1.4). Inclusion of molecular data from other members of Erwin's *americanus* and *cordicollis* groups, which we hypothesize are all members of the revised *cordicollis* species group, would

clarify the relationships within this group. Furthermore, the results of such a phylogenetic analysis would shed light on the evolution of the morphologies in this group.

South American taxa and the lateralis *group*

The *lateralis* species group, and two unidentified species of South American *Neobrachinus*, formed a strong clade in all analyses for which sequences were available (Fig. 3; Table 1). All members of the *lateralis* group possess brown elytra (Fig. 3); this trait is shared with other species described in Erwin that were not in the molecular study, including *B. brunneus*, *B. grandis*, *B. melanarthrus*, *B. sallei* (1970). Notably, the two unidentified South American species have blue elytra, like many other *Neobrachinus* (e.g. Fig. 1f). In molecular analyses, the two South American taxa formed a group sister to the *lateralis* species group (Fig. 3), which could suggest that *Neobrachinus* with brown elytra evolved from a single ancestral species with brown elytra. Examination of other *Neobrachinus* with brown elytra within a molecular phylogenetic context would help determine if this is the case.

In addition to the species examined in Erwin, there are over 20 *incertae sedis* species of *Neobrachinus* primarily from South America that have yet to be examined within a phylogenetic context (Table 2; GBIF.org). Many of these are very poorly sampled, and/or specimens are not publicly available on biodiversity databases (GBIF.org). The lack of research on South American *Neobrachinus* highlights the need for greater sampling for *incertae sedis* taxa, and for phylogenetic work to continue on this group as a whole.

Incertae sedis *taxa*

The results of the molecular phylogeny suggest that molecular data is required to elucidate *Neobrachinus* phylogeny, particularly at the species group level. Therefore, many species and species groups that were not examined in this study have been considered *incertae sedis*. The former *fumans* species group contained a polytomy of seven species that shared the troughed virga with other members of Erwin's *fumans* group, however their morphological distinctiveness warranted their placement in single-species subgroups: e.g. the *oaxacensis* species subgroup only contained *B. oaxacensis*. Only one of these, *B. medius*, had high molecular support for its relationship to other members of *Neobrachinus*; the other six, three with molecular data and three without, remain *incertae sedis*.

In the concatenated analysis, *B. cyanipennis*, *B. gebhardis*, *B. kavanaughi*, *B. mexicanus*, and *B. ovipennis* formed a clade (Fig. 3). However, the single-gene analyses show that this clade was largely driven by COI sequences; 28S and CAD analyses did not recover this group (Figs S1.2, S1.3, S1.4). The uncertain placement of these species warrants their status as *incertae sedis*.

Misidentifications

Molecular phylogenetic analysis revealed several sequences of *Neobrachinus* acquired from BOLD belonged to specimens that are likely misidentified. Some specimens from North Dakota were identified as members of *B. medius* (BETN1837-18, BETN9260-20, BETN9117-20, BETN9121-20), however the sequences were in a clade of the *cordicollis* group (Fig. S1).

Another potential misidentification was found in the *mexicanus* clade. Four specimens, two from *B. ovipennis* and two from *B. cyanipennis*, formed a clade with zero branching and no variance (BS = 1.0) indicating that they likely belong to the same species. Only COI sequences were available for *B. cyanipennis*, while the two specimens of *B. ovipennis* had sequences from COI, CAD, and 28S. It is possible that specimens of either *B. ovipennis* or *B. cyanipennis* were misidentified. *B. ovipennis* is characterized externally by its ovate elytra, and through genitalic characters of the collapsed median lobe and the orientation of virga. *B. cyanipennis* can be distinguished from *B. ovipennis* by its strongly cordate, or heart-shaped, pronotum, and on the elytra the erect depression setae are two-three times as long as the elytral pubescence. Maps of the distributions of these two species show that they are largely sympatric with one another (Erwin 1970).

Considering the challenges of identification to the species level using only morphological characters among *Neobrachinus*, enabling molecular identification by building and contributing to sequence libraries within databases such as BOLD and GenBank would ease these difficulties. Given the high morphological conservation among *Neobrachinus*, it is also possible that cryptic diversity exists within this subgenus; widespread species may harbor cryptic diversity that cannot be described using morphology alone.

Chapter 5: Conclusion

This research presents a molecular test of a previous hypothesis of *Neobrachinus* phylogeny based on morphological characters. The majority of species examined molecularly confirmed the species and species groups posited with morphology by Erwin (1970). However, the polyphyly of the *fumans* group highlights the necessity of using molecular sequence data to elucidate phylogeny, particularly for morphologically challenging taxa. The evolution of morphology and the identification of diagnostic characters can be facilitated by molecular-based trees as demonstrated in this study by the discovery of new apomorphies for new species groups, the *fumans* and *phaeocerus* groups. Finally, the relationships between species groups of *Neobrachinus* remain unclear, although the molecular phylogeny indicates a different scenario of evolution than proposed by Erwin (1970).

This research elucidated a phylogeny of more than half of the species of *Neobrachinus* examined by Erwin; additional taxon sampling, and examination of additional loci to resolve the

phylogeny, would thoroughly test Erwin's hypothesis (1970). Some species in this study were represented by a single specimen due to specimen age and rarity, and require additional material to fully resolve their phylogeny. Pinned specimens for most species that were not in the molecular phylogeny are also very rare and quite old. Furthermore, there are 22 *incertae sedis* species of *Neobrachinus*, all from Central and South America. Targeted sampling to acquire fresh material, particularly of rare species, and increased sampling in Central and South America, would help to provide a clearer picture of the biodiversity of *Neobrachinus*.

This systematic study of *Neobrachinus* emphasizes the importance of continued taxonomic and phylogenetic work in order to better understand the biodiversity of these incredible beetles. Bombardier beetles are abundant members of riparian arthropod communities, particularly in the southwestern U.S. (Moody and Sabo 2017; Schaller et al. 2018) which harbors more than 20 species of *Brachinus*. Strengthening morphological species descriptions with robust molecular support in turn empowers the scientific community with accurate biodiversity inventories and the taxonomic language with which to discuss it. In contrast with what George Ball wrote in 1960, with the molecular tools available to systematists, we are now able to tackle the taxonomic challenge posed by species of bombardier beetles in the Western Hemisphere.

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