Phylogeny of the stink bug tribe Chlorocorini (Heteroptera, Pentatomidae) based on DNA and morphological data

Bruno C. Genevcius[1*a], Caroline Greve[2,3], Samantha Koehler[4], Rebecca B. Simmons[5], David A. Rider[3], Jocelia Grazia[2] & Cristiano F. Schwertner[1,6]

1 – University of São Paulo (USP), Museum of Zoology, São Paulo, SP, Brazil.

2 – Federal University of Rio Grande do Sul (UFRGS), Department of Zoology, Porto Alegre, RS, Brazil.

3 – North Dakota State University (NDSU), Department of Entomology, Fargo, ND, United States of America.

4 - University of Campinas (UNICAMP), Department of Plant Biology, Campinas, SP, Brazil.

5 - University of North Dakota (UND), Department of Biology, Grand Forks, ND, United States of America.

6 – Federal University of São Paulo (UNIFESP), Department of Ecology and Evolutionary Biology, Diadema, SP, Brazil.

** - corresponding author - bgenevcius@gmail.com*

a - current address: University of São Paulo, Department of Genetics and Evolutionary Biology, São Paulo (SP), Brazil.

ABSTRACT

Pentatomidae is the fourth largest family of true bugs, comprising nine subfamilies and over 40 tribes. Few tribes in the family have been studied in a phylogenetic context, and none of them have been examined using molecular data. Here, we conduct a phylogenetic study of the tribe Chlorocorini (Pentatominae) combining 69 morphological characters and five DNA loci in a bayesian framework. The tribe stands out as the most diverse tribe in the subfamily Pentatominae which occurs exclusively in the New World. Chlorocorini was proposed as a probable monophyletic group based on synapomorphies found on nearly all body parts, including the spined humeral angles of the pronotum, a dorsal projection on the apices of each femora, the absence of an abdominal spine and the presence of well-developed, paired projections in the male genitalia (hypandrium). Here, we provide solid evidence that the tribe as currently recognized is not monophyletic based both on DNA and morphological data. The genera *Arvelius* Spinola and *Eludocoris* Thomas were consistently placed outside of the Chlorocorini, while the remaining genera were found to form a monophyletic group. Furthermore, all morphological diagnostic characters for the tribe were homoplastic, except for the developed hypandrium. Lastly, we also provide a preliminary glimpse of main phylogenetic relationships within the Pentatomidae, which indicate that most of the included subfamilies and tribes are not monophyletic. Our results suggest that the current classification of Pentatomidae is not completely adequate to reflect its evolutionary history, and we urge for a complete phylogeny of the family.

Key-words: classification, molecular, Neotropics, phylogenetics, stink bugs, taxonomy

INTRODUCTION

Pentatomidae corresponds to the fourth largest family of true bugs (Hemiptera, Heteroptera). With nearly 5,000 species and over 900 genera, pentatomids are distributed in all terrestrial biomes, except Antarctica (Grazia *et al.*, 2015). They exhibit a plethora of anatomical and behavioral characteristics that make the group interesting models to approach evolutionary and ecological questions. Examples of these features include a variety of feeding habits (Weirauch *et al.*, 2018), aposematism (Paleari, 2013), exaggerated sexual traits (McLain, 1981), and parental care (Requena *et al.*, 2010), among others. However, evolutionary studies addressing these topics are practically unfeasible with pentatomids due to the absence of phylogenetic hypotheses for major groups. While the position of the Pentatomidae was secondarily explored in studies focusing on other pentatomoids (e.g. Wu et al. 2016; Liu et al. 2019), our knowledge about the lineages that compose the family and the relationships among them remain elusive.

The family is currently divided into nine to ten subfamilies, depending on the classification hypothesis (Schuh & Slater, 1995; Grazia *et al.*, 2015; Rider *et al.*, 2018). Most subfamilies are arguably monophyletic as they exhibit sets of unique and remarkable anatomical features not present in any other group (Rider, 2000). For example, the Asopinae show a range of elaborations in the head and mouthparts that most likely represent a single-origin adaptation to their preying habits (Parveen *et al.*, 2015). The exception is the most diverse subfamily, Pentatominae, whose monophyly has been broadly questioned (Grazia *et al.*, 2008a, 2015). The classification within this group has been called “chaotic” (Rider, 2000), currently comprising over 40 tribes that encompass all genera that do not fit in the other subfamilies. Few tribes of Pentatomidae were studied in a phylogenetic context (e.g. Campos and Grazia 2006; Bernardes et al.

2009; Schwertner and Grazia 2012), and none of these utilize molecular data. Therefore, the current classification is mostly based on morphological comparisons of traditional characters whose reliability for identifying natural groups has rarely been assessed. The availability of molecular data for species of Pentatomidae has increased over recent years; however, taxon sampling is extremely biased to Asian and European species (e.g. Yuan et al. 2015; Wu et al. 2016; Liu et al. 2019). Establishing phylogenetic relationships for the pentatomid fauna of the New World is considered paramount for developing a more accurate classification for the family, and for better understanding the evolution of stink bugs.

Among the tribes of Pentatominae, Chlorocorini stands out as the most diverse tribe in the subfamily which occurs exclusively in the New World (Rider *et al.*, 2018). The tribe comprises 77 species organized into eight genera: *Arvelius* Spinola (17 spp.), *Chlorocoris* Spinola (24 spp), *Chloropepla* Stål (14 spp.), *Eludocoris* Thomas (1 sp.), *Fecelia* Stal (4 spp.), *Loxa* Amyot and Serville (10 spp.), *Mayrinia* Horváth (3 spp.), and *Rhyncholepta* Bergroth (4 spp.). *Chlorocoris* is the most diverse genus, and it is the only one devided into sub-genera: *Chlorocoris* (*Arawacoris*), *Chlorocoris* (*Chlorocoris*) and *Chlorocoris* (*Monochrocerus*). Several authors previously suggested close relationships among some of these genera, formerly placing them in the tribe Pentatomini (Becker & Grazia, 1971; Rolston & McDonald, 1984). Stål (1868) described *Chloropepla* and keyed it together with *Chlorocoris* and *Loxa*; later, he also included *Fecelia* in his key (Stål, 1872). More recently, Grazia (1968, 1976) suggested that *Chlorocoris*, *Chloropepla*, *Loxa*, *Mayrinia* and *Fecelia* are related based on the general morphology (e.g. body coloration, head and general body shape, highlighting the presence of spined humeral angles and a dorsal apical projection on each femur as the main diagnostic characters) (Fig. 1); however, these femoral projections are not

found in *Chlorocoris* (Eger, 1978; Thomas, 1985). The genera *Arvelius*, *Eludocoris* and *Rhyncholepta* were later added to the group based on the presence of at least some of the morphological features described as characteristics of the tribe, such as the humeral angles projected and the tapering apexes of the juga (Becker & Grazia, 1971; Thomas, 1992; Greve *et al.*, 2013; Kment *et al.*, 2018; Rider *et al.*, 2018). However, it has been only recently that these eight genera have been formally recognized as a distinct tribe within the Pentatominae (Rider *et al.*, 2018).

The tribe has been considered to be monophyletic based on several characteristics found on nearly all body parts (Greve *et al.*, 2013; Rider *et al.*, 2018). For example, some of the purported defining characters are the triangular head, the spined humeral angles on the pronotum, the dorsal projections on the apices of the femora, the absence of an abdominal spine, and the presence of a well-developed pair of projections (the so called hypandrium) in the male genital capsule. There are also additional features that, in combination, are diagnostic for species of the Chlorocorini: a depressed body, anterior pronotal margins with conspicuous denticles, short ostiolar rugae, and a medially-carinate mesosternum (Becker & Grazia, 1971; Greve *et al.*, 2013; Rider *et al.*, 2018). Nevertheless, it is noteworthy that many of these characteristics are either absent in part of the Chlorocorini species (e.g. the aforementioned apical projection of the femur) or also exhibited by other groups outside of the tribe (e.g. Barcellos & Grazia, 2003; Bernardes *et al.*, 2009). It is therefore unclear if these structures are indeed good indicators of monophyly or are homoplastic and poorly informative for the evolutionary history of these bugs. To address these questions, a phylogenetic study is necessary, preferably using additional sources of information such as the molecular data.

Herein, we conduct a phylogenetic study of the tribe Chlorocorini including representatives of all genera and the three subgenera of *Chlorocoris* within the ingroup taxa. This is the first phylogenetic analysis within the Pentatomidae at the tribal level using a combination of morphological and molecular data. By integrating partial sequences of five molecular markers and 69 morphological characters, we first aimed to test the monophyly of the tribe, recognize lineages within the Chlorocorini and determine its phylogenetic position within the family. Second, we test whether the traditional characters used in taxonomic delimitations within the Pentatomidae are congruent with combined gene trees to recognize monophyletic groups. Lastly, we discuss broader implications of our study and how it might influence the classification of Pentatomidae, highlighting future directions for further understanding the evolution of these important insects.

MATERIAL AND METHODS

Taxon sampling

Our analyses included 37 terminal taxa, comprising as ingroups twelve representatives of the eight genera of Chlorocorini and the three subgenera of *Chlorocoris*. As outgroups, we included 23 species from four subfamilies of Pentatomidae that occur in the Neotropics (Asopinae, Discocephalinae, Edessinae and Pentatominae) and two species of closely related families (Scutelleridae and Acanthosomatidae). Our outgroup choices are carefully designed to include genera that also exhibit some of the characters that have been considered to be synapomorphic within the Chlorocorini, with emphasis placed on the New World fauna.

144 *DNA markers and data aquisition*

145 Genomic DNA was extracted from thoracic muscle of adults previously stored in 100%
146 ethanol and preserved in -80 degrees Celcius freezers. We used the DNeasy Blood &
147 Tissue kit (QIAGEN, Valencia, CA), following the manufacturer's protocol. Partial
148 sequences of five loci were amplified, comprising four ribosomal gene regions (16S
149 rDNA, 18S rDNA, 28S D1 rDNA, 28S D3-D5 rDNA) and one mitochondrial protein-
150 coding gene (COI). Primer sequences used in amplification and sequencing are listed in
151 table S1. All markers were amplified with a single pair of primers, except for the 18S
152 which was amplified using two pairs (Table S1). Polymerase chain reaction (PCR) was
153 used for amplification with the following reagent volumes: 14.2 µl of ddH_2O, 1 µl of
154 dNTP (2.5 mM), 2.5 µl of buffer (10×), 3.0 µl of MgCl2 (25 mM), 1 µl of each primer
155 (10 µM), and 0.3 µl of platinum *Taq* polymerase (2.5 Units) (Invitrogen, USA). PCR
156 programs were the same for all markers (except for the annealing temperature): 94°C
157 for 1 min, 35 cycles of 94°C 30 s, 45–55°C 30 s (Table S1), 72°C 1 min, and 72°C for
158 10 min. Amplification results were visualized though electrophoresis (1% gel agarose)
159 with the SyberSafe gel staining and UV illuminator. Purification DNA sequencing were
160 conducted by Macrogen, Inc (Seoul, South Korea). Sequences were deposited in
161 GenBank (see Table S2 for access codes).

162

163 *Sequence alignment and phylogenetic analyses*

164 Sequences were assembled and edited in GENEIOUS 7.1.7 (Biomatters, Auckland,
165 New Zealand). Alignments were conducted individually in the online v. 7 of MAFFT
166 with the G-INS-I algorithm and default parameters, and further manually checked for

167 inconsistencies. Sequence-matrix 1.8 was used to combine individually aligned
168 molecular markers into a single partitioned dataset.

169 Phylogenetic analyses were conducted using Bayesian Inference (BI). Molecular
170 and morphological datasets were analyzed both separately and in combination in a total
171 evidence partitioned analysis. Different evolutionary models were allowed for each
172 marker, while the Mk model was constrained for the morphological partition. Model
173 selection for DNA markers were run using ModelFinder (Kalyaanamoorthy *et al.*, 2017)
174 through the IQ-TREE software v.1.6.9 (Nguyen *et al.*, 2015), using the BIC criterion
175 and restricting to models available in Mr. Bayes (Ronquist *et al.*, 2012). Phylogenetic
176 analyses were run in Mr. Bayes v.3.2.6 (Ronquist *et al.*, 2012) through the CIPRES web
177 portal (http://www.phylo.org) with the following parameters: 30 million generations,
178 two independent runs, four chains, default priors, and sampling trees every 3000
179 generations. Convergence among runs were checked by confirming that the average
180 standard deviation of split frequencies reached < 0.01, and posterior distributions of
181 parameter estimates were visualized in TRACER v.1.6.0. We discarded the first 10% of
182 the generations as burn-in and constructed a 50% majority rule consensus from the
183 remaining trees. Phylogenetic trees were visualized and edited in FigTree v.1.4.0
184 (http://tree.bio.ed.ac.uk/software/figtree). All matrices and tree files are deposited in
185 TreeBase.

186

187 *Morphological analyses*

188 Morphological characters were either reinterpreted from the literature or described for
189 the first time, totaling 69 morphological features of the whole body: head, thorax,
190 abdomen and the genitalia. Terminology follows Greve et al. (2013) and references

therein. Characters based on literature are listed and indicated in Table 1. Newly described characters were analyzed using a stereo microscope Leica M205C.

We recognized a priori eight morphological characters that have been proposed as key characters for the recognition of species of Chlorocorini (Rider *et al.*, 2018). To analyze the diversification patterns of these characters within Chlorocorini, we conducted a maximum likelihood ancestral state reconstruction using MESQUITE v. 3.0.4. We report the likelihood of the ancestral states at selected branches where evolutionary changes are more likely to have happened. We discuss the relationships within Chlorocorini and between the tribe and other groups with emphasis on these morphological changes.

RESULTS

Evolutionary models for each molecular marker selected in ModelFinder are shown in Table 2. Bayesian analysis with the morphological data alone resulted in a consensus tree with overall poor support, showing several polytomies, especially at deeper nodes (Fig. 2). Although most of the tribes and subfamilies sampled in this study emerged as monophyletic, posterior probabilities were relatively low and relationships among them were poorly resolved in the morphological tree (Fig. 2). The only major groups recognized with high support were the Edessinae (pp = 1) and a group of Neotropical genera currently included in the tribe Carpocorini (pp = 0.95). The tribe Chlorocorini did not emerge as monophyletic because *Arvelius* was placed outside the tribe (Fig. 2).

Analysis of the combined molecular markers showed substantially improved posterior probabilities and overall better resolution than the morphological tree (Fig. 3). In contrast with the morphological analysis, only the Neotropical Carpocorini and the

215 Nezarini were recovered as monophyletic. Both the Edessinae and the Discocephalinae
216 did not emerge as monophyletic, while clade A (pp = 0.91) indicate the subfamily
217 Asopinae as phylogenetically related to species from tribes of Pentatominae (Fig. 3).
218 The monophyly of Chlorocorini had even less support in this analysis (Fig. 3) as
219 *Arvelius*, *Chlorocoris* and *Eludocoris* were phylogenetically associated with other tribes
220 or subfamilies. The position of *Arvelius* was congruent with the morphological analysis,
221 being placed outside of the Chlorocorini, and placed as possibly closely related to
222 *Taurocerus edessoides* (Spinola). However, the position of *Chlorocoris*, which was
223 consistently monophyletic, and *Eludocoris grandis* were incongruent between the
224 morphological and molecular topologies, appearing in the latter as closely related to the
225 Edessinae and *Ocirrhoe*, respectively. The remaining genera currently place in the
226 Chlorocorini formed a monophyletic group as in the morphological analysis, although
227 relationships within this group were significantly different from the morphological
228 analysis (Figs. 2-3).

229 Although morphological and combined gene tree were only partially congruent,
230 the total evidence tree had the highest resolution among the three analyses (Fig. 4). This
231 tree displays relationships found in both morphological and molecular topologies, with
232 more similarities with the molecular tree. Only three clades in the total evidence tree are
233 supported only by morphological evidence (orange rectangles in Fig. 4): Edessinae,
234 *Rhyncholepta* + the remaining Chlorocorini (excluding *Arvelius* and *Eludocoris*) and
235 *Chloropepla* + *Mayrinia* + *Fecelia* + *Loxa*. In contrast, ten clades are supported
236 exclusively by DNA data, for example, the relationships between *Chloropepla* and
237 *Mayrinia* and between *Chlorocoris complanatus* and *Chlorocoris distinctus* (Fig. 4).
238 Relationships at deeper nodes were also improved in the total-evidence tree. For

239 example, the Discocephalinae and *Serdia* Stål (currently placed in the Pentatomini) are
240 strongly supported as the sister lineage of the remaining pentatomids (Fig. 4).

241 The non-monophyly of Chlorocorini was also corroborated by the total-evidence
242 analysis. The tribe emerged, again, as polyphyletic due to the position of *Arvelius* and
243 *Eludocoris* (Fig 4.). *Arvelius* was closely related to *Taurocerus* and other tribes of
244 Pentatominae, while *Eludocoris* was the sister group of Edessinae + *Myota* + *Ocirrhoe*.
245 The remaining genera formed a well-supported monophyletic group, which could be
246 split into three groups: the three subgenera of *Chlorocoris*, the genus *Rhyncholepta*, and
247 the clade *Loxa* + *Fecelia* + *Mayrinia* + *Chloropepla*. The sister group of Chlorocorini
248 (excluding *Arvelius* and *Eludocoris*), which was ambiguous in the separated analyses of
249 each type of data (morphological versus molecular), was the Edessinae plus *Myota*
250 Spinola and *Eludocoris.* This relationship had moderate support.

251 Reconstructed ancestral states of selected characters revealed that all genera of
252 the Chlorocorini (excluding *Arvelius* and *Eludocoris*) share several synapomorphies
253 (characters 3^1, 5^1, 22^1, 26^0, 34^1, 57^1 and 65^1). These synapomorphies include characters
254 of the head, thorax, abdomen and genitalia (Fig. 5), such as a long ostiolar ruga
255 (character 26^0), dorsal apical projections in the femora (34^1) and a well-developed
256 conjunctiva (65^1). The position of *Arvelius* outside of Chlorocorini is also supported by
257 numerous morphological characters, for example, a medium ostiolar ruga (26^1), the
258 absence of the hympandrium (57^0) and an undeveloped conjunctiva (65^0). In contrast,
259 *Eludocoris* has retained plesiomorphic conditions for most of the diagnostic characters
260 of the Chlorocorini. For instance, the genus presents a short mandibular plate and a
261 short ostiolar ruga. The position of the genusis primarily supported by the molecular
262 data (Fig. 4).

263

264

265 **DISCUSSION**

266 *Monophyly of Chlorocorini*

267 Our study proposes the first phylogenetic hypothesis for subfamilies and tribes of
268 Pentatomidae using both molecular and morphological data. The morphological data
269 analyzed by itself provided good resolution for families and tribes, but also left the
270 backbone of the tree mostly unresolved (Fig. 2). In contrast, the combined gene tree has
271 much better resolution from tips to the root, even though many clades showed modest to
272 poor posterior probabilities (Fig. 3).

273 Our analyses refuted the hypothesis of monophyly for the eight included genera
274 of Chlorocorini currently recognized by Rider et al. (2018). Two genera were
275 consistently placed out of the tribe: *Arvelius* and *Eludocoris*. It should be noted that
276 (Rider *et al.*, 2018) indicated that *Arvelius* might not belong in this tribe, based on
277 morphological characters, especially based on the armed nature of the abdominal base.
278 The polyphyly of Chlorocorini is maintained whether molecular and morphological data
279 are analyzed individually or separately on their own (Fig. 2-4). In all trees, *Arvelius* was
280 positioned outside of Chlorocorini. The genus consistently emerged as the sister group
281 of *Taurocerus* Amyot & Serville*;* both were more closely related to other genera of
282 Pentatominae than to genera of Chlorocorini. This result is not altogether surprising as
283 both Brailovsky (1981) and Grazia & Barcellos (2005) highlighted similarities between
284 the two genera, including the apical projection in each femur, the abdominal spine
285 present and opposed to the metasternum, and the morphological aspect of the phallus.
286 The genus *Eludocoris* also contributed to the polyphyly of the tribe, yet its current

287 position was unstable among all resulting topologies. According to Thomas (1992),
288 *Eludocoris* should be placed near *Loxa* and allied genera (*Chlorocoris*, *Chloropepla* and
289 *Fecelia*) based primarily on the large body size, the elongate and depressed body shape,
290 the greenish coloration when alive, and the presence of an apical projection on each
291 femur. This hypothesis is in agreement with our morphological topology, as *Eludocoris*
292 is the sister group of the remaining genera of Chlorocorini. On the other hand, the
293 molecular data indicates that *Eludocoris* may be closely related to the Australian genus
294 *Ocirrhoe* (which is currently placed in the tribe Rhynchocorini), and both genera were
295 the sister group of the remaining Pentatominae. In the total-evidence analysis,
296 *Eludocoris* is associated with *Myota* and the Edessinae. It is obvious, however, that
297 these possible placements are tentative at best, and further studies are needed.

298 Our outgroup choice provided a robust sample that is representative of the
299 Neotropical lineages which also exhibit the diagnostic features of the Chlorocorini. This
300 careful outgroup choice enabled us to confidently investigate both the monophyly of the
301 Chlorocorini and also the amount of homoplasy found in traditional diagnostic features.
302 We show that all characters that group the genera of Chlorocorini in the taxonomic
303 literature (Becker & Grazia, 1971; Grazia *et al.*, 2008b; Rider *et al.*, 2018) have some
304 level of homoplasy, with the exception of the development of the hypandrium (Fig. 5;
305 character 57[1]). One of the most homoplastic characters was the length of the mandibular
306 plates, which changed from short to long four times independently across the total
307 evidence phylogeny (Fig. 5). In fact, relatively high levels of homoplasy for characters
308 describing head shape and genital development are not altogether surprising, as these
309 have been shown homoplastic in many other phylogenetic studies within Pentatomidae
310 (e.g. Genevcius and Schwertner 2014; Weiler et al. 2016; Bianchi et al. 2017). One
311 likely reason for high levels of homoplasy is that these characters are arbitrary

312 categories of structures with continuous variation; therefore, states for these characters
313 tend to overlap (Cohen, 2012). Most of the diagnostic characters for the Chlorocorini
314 are likely artificial constructs, for example, the shape of mandibular plates (character 7),
315 the length of the ostiolar ruga (character 26), and the degree of development of the
316 conjunctiva (character 65). These results call the reliability of these characters into
317 question, especially as used to propose a classification of the Pentatomidae based on the
318 evolutionary history of the group. Further phylogenetic and taxonomic studies should
319 take into account that these characters are intrinsically continuous, and their use should
320 be considered with caution.

321

322 *Position of Chlorocorini and relationships among genera*

323 While the non-monophyly of Chlorocorini as currently recognized was strongly
324 supported, the position of its genera among the other lineages of the Pentatomidae was
325 less consistent. The morphological tree exhibited a large, basal polytomy that did not
326 provide the certainty for identifying the sister group of Chlorocorini. In the molecular
327 analysis, the genus *Chlorocoris* was closely related to the Edessinae, while the
328 remaining genera (except *Arvelius*) were the sister group of the Pentatominae (including
329 Asopinae). Lastly, results of the combined analysis were partially congruent with the
330 molecular analysis. Accordingly, all genera of Chlorocorini (except *Arvelius*) were
331 grouped with the Edessinae, and this clade was the sister lineage of the remaining
332 Pentatominae.

333 Although the position of Chlorocorini was poorly supported with either DNA or
334 morphological data analyzed individually, there were some consistent relationships
335 between the two datasets. None of the genera (except *Arvelius*) were placed within the

336 Pentatominae, which indicates no for including the Chlorocorini as a tribe of
337 Pentatominae, as in the current classification of the family (Greve *et al.*, 2013; Rider *et*
338 *al.*, 2018). We conclude that the genera *Chlorocoris*, *Chloropepla*, *Fecelia*, *Loxa*,
339 *Mayrinia* and *Rhyncholepta* indeed compose a distinct evolutionary lineage. This group
340 should either comprise a new subfamily or be incorporated with *Eludocoris* to the
341 subfamily Edessinae. We do not seek to formally propose one of these changes here
342 because of the poor resolution of these clades and because our taxon sampling is limited
343 (especially with respect to the European, Asiatic and Australian faunas). Furthermore,
344 there is not a single morphological synapomorphy to the clade comprising the Edessinae
345 and the Chlorocorini, which would require closer inspection of the morphology
346 focusing on an alternative level of analysis. Therefore, to allow for such taxonomic
347 decisions, we urge for a complete phylogeny of the Pentatomidae with datasets
348 including greater amount of morphological and molecular data.

349 Overall, groupings within Chlorocorini were more stable than the position of the
350 tribe itself within the subfamily. Furthermore, some relationships among genera are in
351 close agreement with the literature, suggesting the traditional taxonomic characters are
352 more reliable at the generic level. All analyses supported the monophyly of the genera
353 *Arvelius*, *Chlorocoris* and *Loxa*, as well as the monophyletic group *Fecelia* + *Loxa*. The
354 latter was also consistently associated with *Chloropepla* and *Mayrinia*, although
355 *Rhyncholepta* may also fall within this group according to DNA data (Fig. 3). The
356 association among *Chloropepla*, *Fecelia*, *Loxa* and *Mayrinia* was not surprising as it
357 has been repeatedly suggested in the literature based on shared features of head,
358 pronotum and scutellum shape (Grazia, 1972; Rolston & McDonald, 1984; Greve *et al.*,
359 2013). The inconsistent position of *Rhyncholepta* may be explained by its highly
360 autapomorphic features. The genus possesses several unique characteristics in the tribe,

361 such as the body color (after death), longer antennae, larger eyes, presence of an
362 abdominal tubercle, and several genital features (Becker & Grazia, 1971; Kment *et al.*,
363 2018). Some of these characteristics have been included in our analyses (e.g. characters
364 35, 57), which may have supported its position as sister of the remaining species within
365 Chlorocorini.

366

367 *Implications for the Pentatomidae classification and future directions*

368 Although not the primary focus of our work, our results also provide a preliminary
369 glimpse of potential major relationships within the Pentatomidae. In agreement with
370 previous studies based on morphology (Gapud, 1991; Thomas, 1992) and DNA (Wu *et*
371 *al.*, 2016; Liu *et al.*, 2019), our study corroborate the monophyly and the position of the
372 Asopinae within of Pentatominae (Fig. 4). Edessinae was also found to be monophyletic,
373 while Pentatominae was broadly polyphyletic. This is not surprising given that the
374 Pentatominae is a "catch-all" construct that encompasses all genera not placed in the
375 other subfamilies (Rider *et al.*, 2018).

376 The Discocephalinae can be considered monophyletic only with the inclusion of
377 *Serdia concolor* Ruckes, representing the sister lineage of all remaining Pentatomidae.
378 Although these conclusions are based on too small of a sample size, the results included
379 herein indicate that the classification of the Pentatomidae at the subfamily and tribal
380 levels will require a thorough reformulation. For example, the tentative results obtained
381 in this study with respect to the whole family indicate that the subfamily Pentatominae
382 may be maintained, but only with the inclusion of the asopines as a tribe and with the
383 exclusion of Chlorocorini and some other genera. On the other hand, it is possible that
384 the Chlorocorini should be raised to a new subfamily, or it could be transferred to the

385 Edessinae. Most of the other included tribes included (i.e. Nezarini, Carpocorini,
386 Catacanthini [*Runibia perspicua* (Fabricius)] and Piezodorini [*Piezodorus guildinii*
387 (Westwood)]), would remain with the new concept of the Pentatominae.

388 In summary, we provide solid evidence that the currently recognized tribe
389 Chlorocorini and two of the subfamilies of Pentatomidae are not monophyletic. Thus,
390 our results indicate that the current classification of the Pentatomidae (sensu Rider et al.
391 2018) does not accurately reflect the evolutionary history in the group. The most likely
392 reason for that is that the traditional diagnostic characters, many of which are based on
393 continuous variation, show considerable levels of homoplasy. Nevertheless, we
394 emphasize that our study was focused on determining the monophyly and the inside
395 relationships within the tribe Chlorocorini. Thus, our findings regarding the major
396 relationships within the entire family should be considered, at best, preliminary. It
397 appears that the molecular data at times supports previous morphological studies, but
398 often it is not in congruence. In these cases, we should re-analyze the morphological
399 data, looking for convergent, parallel, and other misinterpretations of the morphological
400 characters. For next steps, we encourage a thorough and representative revision seeking
401 to find synapomorphies for early diverging lineages, whose phylogenetic placements
402 were poorly supported in our study. Although the inclusion of molecular data improved
403 phylogenetic resolution overall, many clades, especially those that were unresolved in
404 the morphological analysis, still had low support. These suggest that the inclusion of
405 additional nuclear DNA data possessing low divergence rates will be fundamental to
406 improve phylogenetic understanding of these lineages. Besides the addition of
407 morphological and DNA data, a reliable classification for the Pentatomidae will only be
408 feasible with a robust taxon sampling scheme representing the global diversity of these
409 insects.

Updated classification of Chlorocorini

Based on our phylogenetic results, we provide a reclassification of the tribe Chlorocorini, now including six genera and 59 species (see below). Provisionally, we suggest that the genus *Arvelius* should tentatively be transferred to the tribe Pentatomini (currently the classification of the related genus *Taurocerus*). The genus *Eludocoris* will be considered unplaced owing to its inconsistent position among the three analyses (Figs. 2-4).

<u>Checklist of the genera and species of Chlorocorini:</u>

Tribe Chlorocorini Rider, Greve, Schwertner and Grazia, 2018

Chlorocoris Spinola, 1837
Type species: *Chlorocoris tau* Spinola, 1837, by monotypy.

Chlorocoris (*Chlorocoris*) *tau* Spinola, 1837 [BRA, ARG, URU]
Chlorocoris (*Chlorocoris*) *depressus* (Fabricius, 1803) [COL, VEZ, SUR, BRA, TTO, ECU]
Chlorocoris (*Chlorocoris*) *complanatus* (Guérin-Méneville, 1831) [BRA, BOL, PAR, ARG, URU]
Chlorocoris (*Chlorocoris*) *deplanatus* (Herrich-Schäffer, 1842) [BRA]
Chlorocoris (*Chlorocoris*) *distinctus* Signoret, 1851 [USA, MEX, BLZ, NIC, HON, GTM, CR, PAN, COL, ECU]
Chlorocoris (*Chlorocoris*) *sanguinursus* Thomas, 1985 [SUR, PER, BOL]
Chlorocoris (*Chlorocoris*) *fabulosus* Thomas, 1985 [BRA]
Chlorocoris (*Chlorocoris*) *humeralis* Thomas, 1985 [BOL]
Chlorocoris (*Chlorocoris*) *isthmus* Thomas, 1985 [CR, PAN, COL, ECU]
Chlorocoris (*Chlorocoris*) *vandoesburgi* Thomas, 1985 [SUR]
Chlorocoris (*Chlorocoris*) *sororis* Thomas, 1985 [COL]
Chlorocoris (*Chlorocoris*) *tibialis* Thomas, 1985 [BRA]
Chlorocoris (*Monochrocerus*) *rufispinus* Dallas, 1851 [MEX, GTM, HON, CR, PAN]
Chlorocoris (*Monochrocerus*) *rufopictus* Walker, 1868 [MEX]
Chlorocoris (*Monochrocerus*) *subrugosus* Stål, 1872 [USA, MEX]
Chlorocoris (*Monochrocerus*) *championi* Distant, 1880 [GTM, MEX]

Chlorocoris (*Monochrocerus*) *irroratus* Distant, 1880 [MEX]
Chlorocoris (*Monochrocerus*) *hebetatus* Distant, 1890 [USA, MEX]
Chlorocoris (*Monochrocerus*) *flaviviridis* Barber, 1914 [USA]
Chlorocoris (*Monochrocerus*) *loxoides* Thomas, 1985 [MEX, GTM, NIC]
Chlorocoris (*Monochrocerus*) *werneri* Thomas, 1985 [USA, MEX]
Chlorocoris (*Monochrocerus*) *biconicus* Thomas, 1985 [NIC, CR, PAN, HON]
Chlorocoris (*Arawacoris*) *tarsalis* Thomas, 1998 [JAM]

Chloropepla Stål, 1868
Type species: *Loxa vigens* Stål, 1860, by original designation.

Chloropepla rideri Greve, Schwertner & Grazia, 2013 [BRA]
Chloropepla paveli Grazia, Schwertner & Greve, 2008 [BRA, BOL]
Chloropepla vigens (Stål, 1860) [BRA, ARG, URU]
Chloropepla luteipennis (Westwood, 1837) [BRA]
Chloropepla lenti Grazia, 1968 [VEZ, HON]
Chloropepla costaricensis Greve, Schwertner & Grazia, 2013 [CR]
Chloropepla aurea (Pirán, 1963) [BRA, PER, BOL]
Chloropepla pirani Grazia-Vieira, 1971 [BOL]
Chloropepla dollingi Grazia, 1987 [GUY, BRA]
Chloropepla tucuruiensis Grazia & Teradaira, 1980 [BRA]
Chloropepla stysi Grazia, Schwertner & Greve, 2008 [BRA, ECU]
Chloropepla rolstoni Grazia-Viera, 1973 [FG, BRA, BOL]
Chloropepla caxiuanensis Greve, Schwertner & Grazia, 2013 [VEZ, BRA]

Fecelia Stål, 1872
Type species: *Loxa minor* Vollenhoven, 1868, by monotypy.

Fecelia minor (Vollenhoven, 1868) [PUR]
Fecelia nigridens (Walker, 1867) [HAI, DRE, TTO]
Fecelia proxima Grazia, 1980 [DRE, TTO]
Fecelia biorbis Eger, 1980 [HAI, DRE]

Loxa Amyot & Serville, 1843
Type species: *Cimex flavicollis* Drury, 1773, by subsequent designation (Kirkaldy, 1903)

Loxa flavicollis (Drury, 1773) [USA, MEX, CUB, JAM, CR, HON, BRA, ECU, FG, TTO]
Loxa virescens Amyot & Serville, 1843 [MEX, HON, NIC, PAN, VEZ, SUR, FGU, BRA, ARG, CR, COL]
Loxa peruviensis Eger, 1978 [PER]
Loxa melanita Eger, 1978 [GUY, BRA, PER]

Loxa deducta (Walker, 1867) [PAN, VEZ, BRA, CHI, BOL, PAR, ARG, URU]
Loxa viridis (Palisot de Beauvois, 1811) [USA, MEX, HON, NIC, DRE, PAN, VEZ, FGU, BRA, ECU, ARG, URU, CR, HAI, CUB, JAM, COL]
Loxa parapallida Eger, 1978 [PER]
Loxa pallida Van Duzee, 1907 [CUB, DRE, BAH, PUR, JAM, DOM]
Loxa planiceps Horváth, 1925 [DRE]
Loxa nesiotes Horváth, 1925 [CUR, GRE, SLU, PAN, COL, VEZ, GUY, DRE, HAI, SVG]

Mayrinia Horváth, 1925
Type species: *Loxa curvidens* Mayr, 1864, by original designation.

Mayrinia curvidens (Mayr, 1864) [BRA, BOL, PAR, ARG]
Mayrinia rectidens (Mayr,1868) [BRA, PER]
Mayrinia variegata (Distant, 1880) [NIC, CR, COL, VEZ, GUY, BRA, PER, MEX, HON]
Mayrinia brevispina Grazia-Vieira, 1973 [PER, BOL, BRA]

Rhyncholepta Bergroth, 1911
Type species: *Rhyncholepta grandicallosa* Bergroth, 1911, by monotypy.

Rhyncholepta grandicallosa Bergroth, 1911 [PAN, VEZ, FG, BRA, CR, HON, COL]
Rhyncholepta henryi Kment, Eger & Rider, 2018 [FG]
Rhyncholepta meinanderi Becker & Grazia, 1971 [VEZ, BOL, BRA]
Rhyncholepta wheeleri Kment, Eger & Rider, 2018 [GUY]

*abbreviations: ARG (Argentina), BOL (Bolivia), BLZ (Belize), BRA (Brazil), CHI (Chile), COL (Colombia), CR (Costa Rica), CUB (Cuba), CUR (Curaçao), DRE (Dominican Republic), ECU (Ecuador), FG (French Guyana), GRE (Grenadines), GUY (Guyana), GTM (Guatemala), HAI (Haiti), HON (Honduras), JAM (Jamaica) MEX (Mexico), NIC (Nicaragua), PAN (Panama), PAR (Paraguay), PER (Peru), SVG (Saint Vincent and the Grenadines), PUR (Puerto Rico), SLU (Saint Lucia), SUR (Suriname), URU (Uruguay), USA (United States of America), TTO (Trinindad & Tobago), VEZ (Venezuela)

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TABLES

Table 1. Morphological characters with respective references (except for new characters).

N	Character and state descriptions
1	Body shape (Rider et al 2018): (0) not depressed; (1) slightly depressed; (2) ventrally depressed (dorsally convex)
2	Surface of body, punctuation (Packauskas & Schaefer 1998, Rider et al 2018): (0) moderate punctuation; (1) rare punctures; (2) strongly marked punctures
3	Body color (Rider et al, 2018): (0) evenly dark (mostly brow/black); (1) evenly green in life, and retaining green coloration after death; (2) yellow-greenish in life, fading to yellow after death; (3) with colorful spots along the dorsum
4	Dorsal surface of head, punctuation (Rider et al 2018): (0) punctuation regularly distributed; (1) nearly non-punctuate, with oblique transverse ridges or wrinkles; (2) nearly non-punctuate, without oblique transverse ridges or wrinkles
5	Head, margin before eyes: (0) concave; (1) straight; (2) convex
6	Apex of mandibular plates beyond tylus: (0) straight forward; (1) curving towards apex of tylus
7	Length of mandibular plates: (0) longer than tylus; (1) shorter than tylus
8	Apex of mandibular plates, shape: (0) truncated; (1) pointed; (2) rounded
9	Mandibular plates, acute process on the apex: (0) absent; (1) present
10	Maxillary plates with (1+1) acute processes: (0) absent; (1) present
11	Distance of labial base from buccular anterior margin (Gapud 1991): (0) distinctly remote from anterior limit of buccula; (1) slightly to moderately closer to anterior limit of buccula; (2) strongly and closely associated with anterior limit of bucculae
12	Posterior angles of the bucculae (Rider et al 2018): (0) evanescent, not projected or truncated; (1) truncated; (2) lobed
13	Distance of labral base from labial base (Gapud 1991): (0) distinctly remote; (1) slightly to moderate closer, but not contiguos; (2) distinctly contiguous
14	Labial development (Gapud 1991): (0) slender; (1) distinctly incrassate, robust
15	Bucculae, shape (Rider et al 2018): (0) straight; (1) flap-like
16	Anterior angle of buccula (Greve et al 2016): (0) evanescent; (1) truncate; (2) with an acute process
17	First rostral segment (Gapud 1991): (0) shorter to subequal than buccula; (1) longer than buccula
18	Antenniferous tubercle ventral lobe: (0) absent; (1) present
19	Length of the first antennal segment related to head apex (Gapud 1991): (0) strongly exceeding head apex (most of the segment); (1) slightly exceeding head apex; (2) short, not reaching head apex
20	Length of scutellum (Gapud 1991, Hasan and Kitching 1993, Grazia et al 2008a): (0) short, not or slightly surpassing posterior margin of metathorax; (1) reaching or surpassing connexivum apical angles of 3rd abdominal segment; (2) extending beyond abdominal segment V but not exceeding VI; (3) long, almost attaining apex of abdomen, but not covering most of the abdomen; (4) covering mostly of abdominal dorsum
21	Lenght of frena (Gapup 1991; Grazia et al 2008a): (0) long, attaining or distinctly surpassing middle of scutellum; (1) short, not surpassing middle of scutellum; (2) obsolete to almost invisible
22	Antero-lateral margins of pronotum, crenulation: (0) anterior half crenulated; (1) entirely crenulated; (2) not crenulated
23	Anterior margins of pronotum reflexed (Rider et al 2018): (0) absent; (1) present
24	Humeral angles, shape (Gapud 1991): (0) developed into a spine; (1) triangular, acute but never produced into processes; (2) rounded; (3) produced into a flat bifid process; (4) produced into a clavate process
25	Posterior margin of pronotum: (0) concave; (1) straight
26	Ostiolar ruga, length (Rider et al 2018): (0) long, reaching lateral third of metapleura; (1) auricular, medium, not surpassing half of the metapleura; (2) straight and short, not reaching the mid length of metapleura
27	Ostiolar ruga, development (Rider et al 2018): (0) with sides and apex distinct; (1) distally effaced

28 Prosternum sulcus, development (Grazia et al 2008a, Rider et al 2018): (0) without sulcus to moderately sulcate; (1) deeply sulcate

29 Mesosternum carina, development: (0) not developed; (1) developed with a low carina; (2) well developed with strong carina

30 Metasternum process, presence (Rolston & McDonald 1979, Barcellos and Grazia 2003, Rider et al 2018): (0) absent; (1) present

31 Metasternum process, development (Barcellos and Grazia 2003): (0) never projected over mesosternum; (1) projected over the mesosternum

32 Mesosternum carina, shape (Gapud 1991, Rider et al 2018): (0) plateau shaped, anteriorly produced; (1) keel shaped, same height the entire length, well developed; (2) keel shaped, same height the entire length, weakly developed (3) keel shaped, apical third projected anteriorly, weakly developed; (4) not developed

33 Metasternum produced: (0) present; (1) absent

34 Femur, dorsal apical projection (Bernardes et al 2009; Schwertner & Grazia 2012): (0) not developed; (1) present, acute; (2) present, obtuse;

35 Abdominal spine, development (Gapud 1991, Rider et al 2018): (0) not developed; (1) well developed, reaching hind coxae; (2) well developed, reaching middle coxae; (3) developed, reaching fore coxae; (4) weakly developemt, just a bump

36 Abdominal venter, longitudinal sulcus (Memon et al 2011): (0) absent; (1) present

37 Anterior margin of urosternite VII in males: (0) convex, not strongly extended anteriorly; (1) strongly extended anteriorly

38 Development of the female external genitalia (Rider et al 2018): (0) all plates well developed and external; (1) gonocoxites VIII reduced, shorter than laterotergite IX in lenght; (2) all plates are reduced and appear to be recessed into female urosternite VII

39 Laterotergites IX, length: (0) long, surpassing the band uniting laterotergites VIII; (1) short, not surpassing

40 Laterotergites IX, apex, shape: (0) rounded; (1) pointed

41 Laterotergites VIII, median pointed process: (0) present; (1) absent

42 Gonapophyses VIII and first rami, development (Grazia et al 2008a): (0) well developed, and distinct; (1) not developed

43 Gonocoxites IX (Gapud 1991, Grazia et al 2008a): (0) with a distinct apparent median fusion line; (1) completely fused with gonapophyses IX

44 Gonapophyses IX (Grazia et al 2008a): (0) moderately sclerotized to membranous, second rami present; (1) reduced, fused to gonocoxites IX, second rami lost

45 Secondary thickning of gonapohysis (STG) IX (Schwertner 2005): (0) small, occupying less than half of the STG; (1) large, occupying more than half of the STG

46 Gonangulum development (Gapud 1991, Grazia et al 2008): (0) well developed, sclerotized; (1) membranous, obsolete; (2) not developed

47 Spermathecal bulb, middle constriction, development (Rider et al 2018): (0) not developed; (1) slightlyb developed; (2) well developed and acuminated

48 Development of ductus receptaculi (Gapud 1991, Grazia et al 2008): (0) not invaginated; (1) invaginated, with distal part open; (2) invaginated, with distal part closed

49 Triangulin aspect [= membranous to sclerotized structure joining the gonocoxites VIII or the gonapophyses VIII] (Gapud 1991, Grazia et al 2008): (0) pleated; (1) smooth

50 Pars communis, development (Grazia et al 2008a): (0) areas surrounding orificium receptaculi largely membranous; (1) areas surrounding orificium receptaculi with elongate, grooved sclerite; (2) areas surrounding orificium receptaculi with a pair of sclerites

51 Capsula seminalis, development of processes (Barcellos & Grazia 2003): (0) not developed; (1) developed and elongated anteriorly; (2) developed with apical arc shape process; (3) developed with apical finger-like process

52 Capsula seminalis, proximal region: (0) plate-shaped; (1) ring or tube shaped; (2) reduced

53 Sternite VIII in males, development (Grazia et al 2008a): (0) apparent externally, not or slightly covered by segment VII; (1) concealed by segment VII

54 Tergite VIII in males, sclerotization (Gapud 1991; Grazia et al 2008a): (0) sclerotized; (1) membranous

55 Pygophore, processes of dorsal rim: (0) present; (1) absent

56 Pygophore, superior processes, presence (Barcellos & Grazia 2003): (0) absent; (1) present

57 Hypandrium, development: (0) not developed; (1) developed into 1+1 broad and long inflated expansions (surpasing the ventral rim); (2) developed into 1+1 flat and long expansions (surpasing ventral rim); (3) slightly developed into 1+1 short expansions

58 Pygophore, ventral wall, median projection: (0) poorly developed; (1) developed apically; (2) developed laterally

59 Segment X apical processes, development: (0) not developed; (1) developed anteriorly; (2) developed posteriorly

60 Paramere, shape: (0) nearly cylindrical, without wide processes; (1) laminar, with wide processes; (2) poorly developed

61 Paramere, connection to the ventral wall: (0) absent; (1) present

62 Paramere, basal process: (0) absent; (1) present

63 Phallotheca, scletorization (Gapud 1991, Grazia et al 2008a): (0) slightly sclerotized; (1) moderately sclerotized, relatively flexible; (2) thickly sclerotized, rigid

64 Thecal shield (Gapud 1991, Hassan & Kitiching 1992): (0) absent; (1) present

65 Conjunctiva, development (Gapud 1991): (0) not developed; (1) well developed; (2) slightly developed

66 Phallus, titilators, presence (Baker 1931): (0) absent; (1) present

67 Conjuctiva, lappet process, presence (Gross 1976): (0) absent; (1) present

68 Vesica lenght in relation to the phallotheca: (0) shorter than phallotheca; (1) subequal to phallotheca; (2) longer than phallotheca

69 Vesica, basal process, presence: (0) absent; (1) present

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Table 2. Alignment sizes and results of model selection from ModelFinder to each molecular marker and morphology.

	Alignment size	Model
16S rDNA	596	HKY+I+G
18S rDNA	2296	K2P+I
28S D3-D5 rDNA	550	K2P
28S D1 rDNA	386	K2P
COI	656	GTR+I+G
Morphology	69	MK

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FIGURES

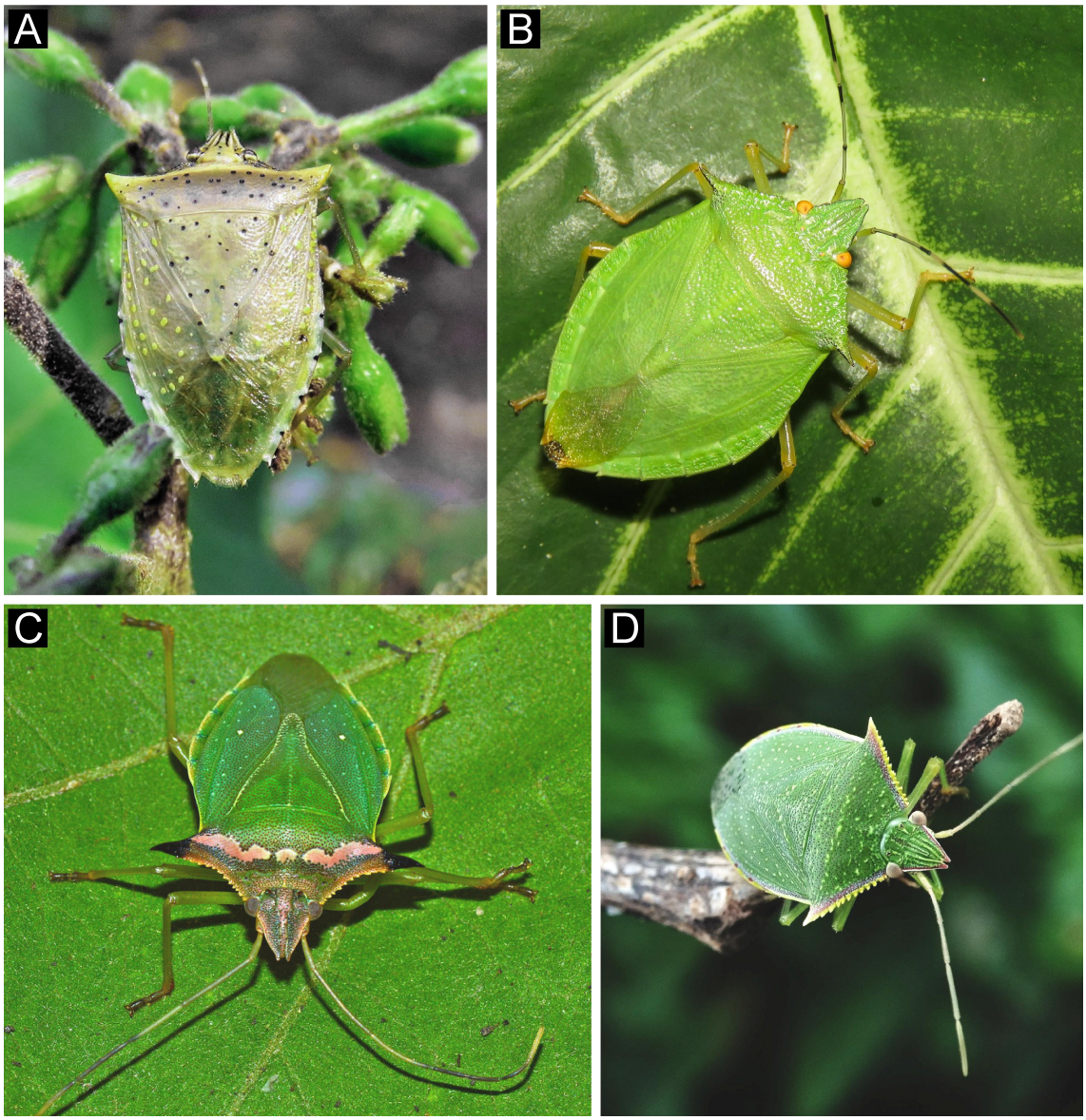

Figure 1. Examples of morphological diversity of the Chlorocorini. (A) *Arvelius albopunctatus* (DeGeer) (photo by Roger Rios Dias), (B) *Chlorocoris complanatus* (Guérin-Méneville) (photo by Diogo Luiz), (C) *Fecelia nigridens* (Walker) (photo by Francisco Alba Suriel) and *Loxa sp.* (photo by Maurino André).

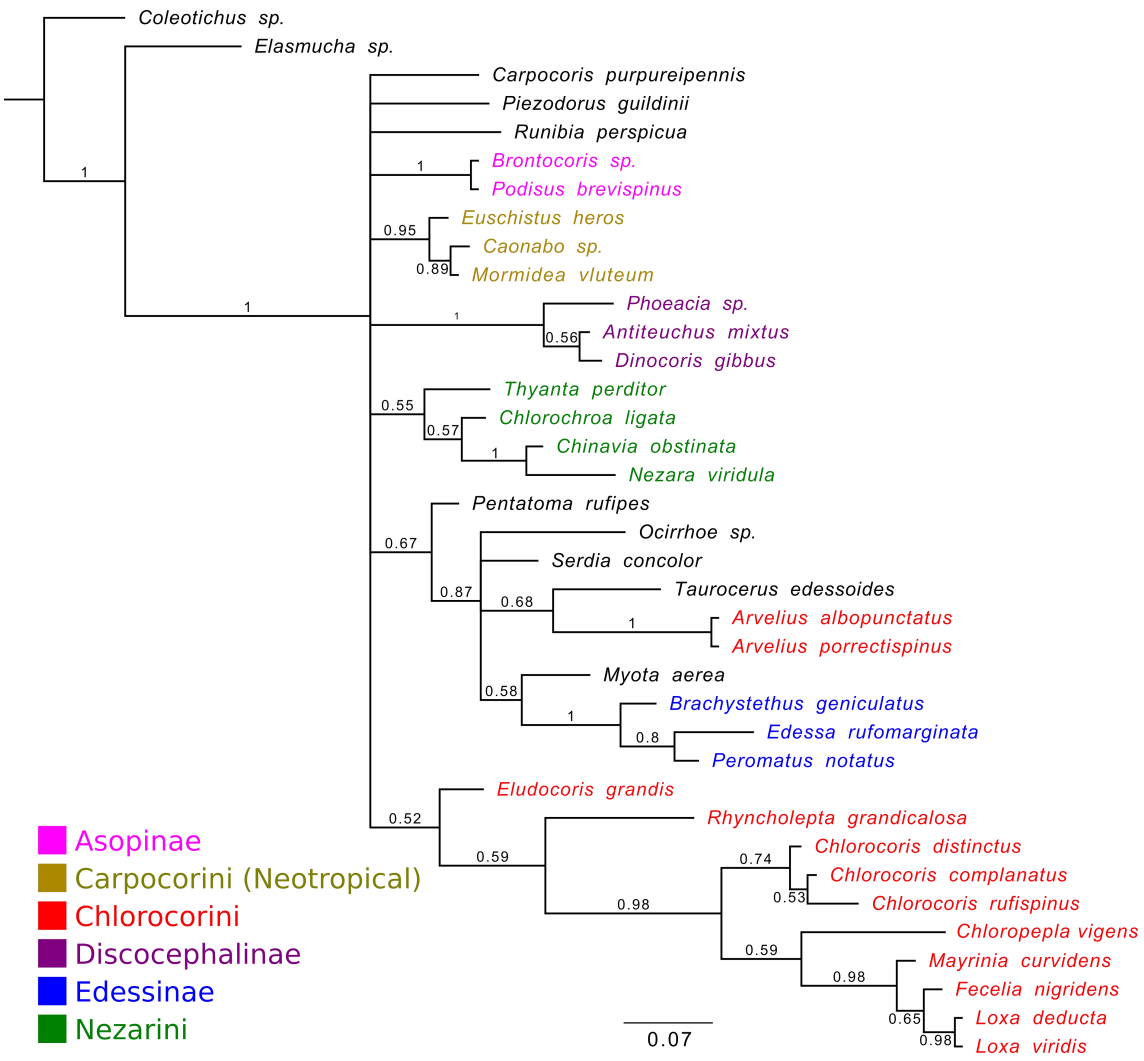

Figure 2. Bayesian majority consensus tree constructed from the 69 morphological characters. Numbers above branches are posterior probabilities.

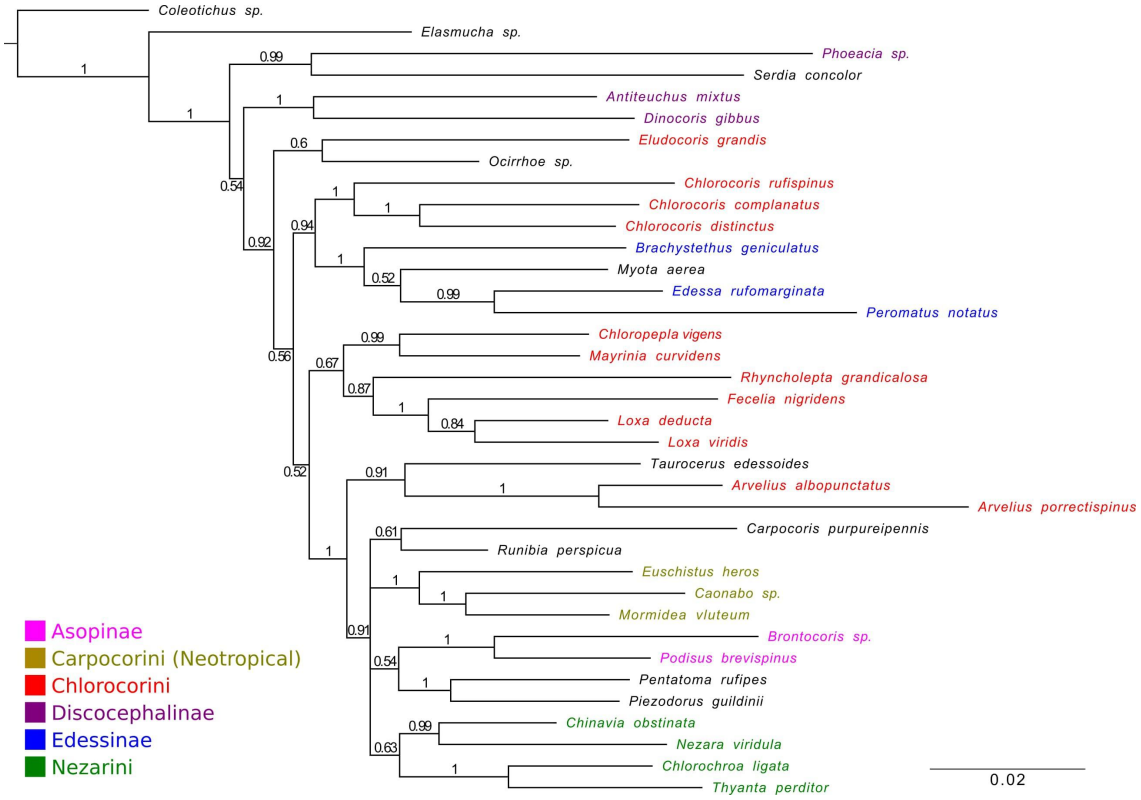

Figure 3. Bayesian majority consensus tree constructed using five molecular markers in combination: 16S rDNA, 18S rDNA, 28S D1 rDNA, 28S D3-D5 rDNA and COI mitDNA. Numbers above branches are posterior probabilities.

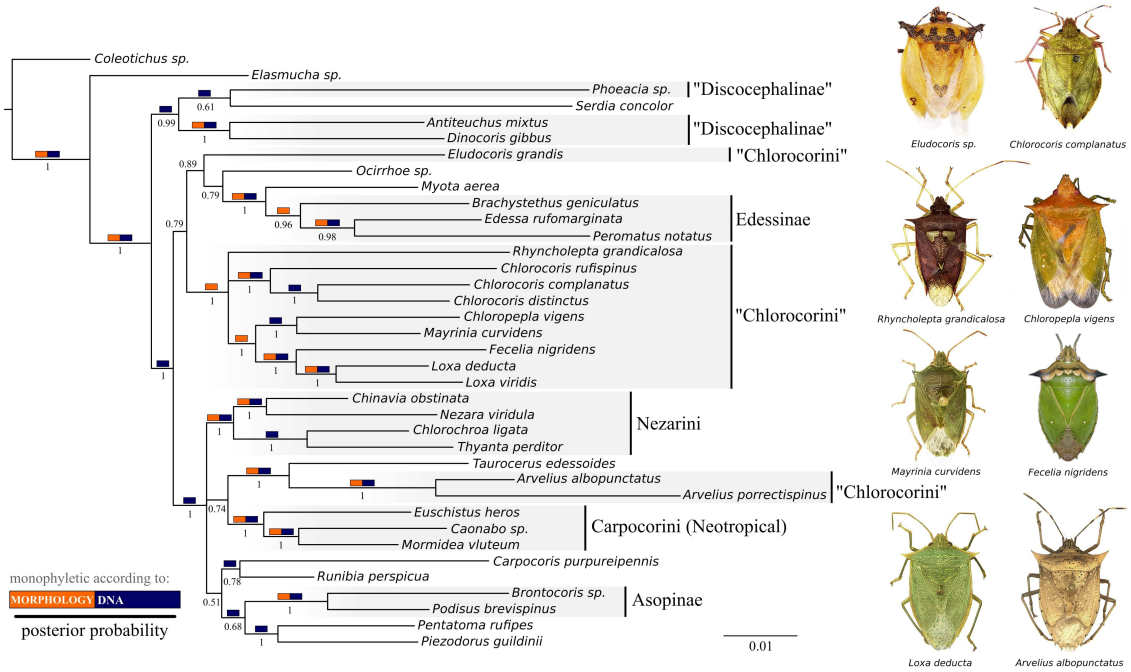

Figure 4. Bayesian majority consensus tree constructed using combined morphological (69 characters) and DNA (16S rDNA, 18S rDNA, 28S D1 rDNA, 28S D3-D5 rDNA and COI mitDNA) data, along with the dorsal habitus of all genera of Chlorocorini. Rectangles above branches indicate whether the clade is supported by morphological and/or molecular data, while numbers below branches are posterior probabilities.

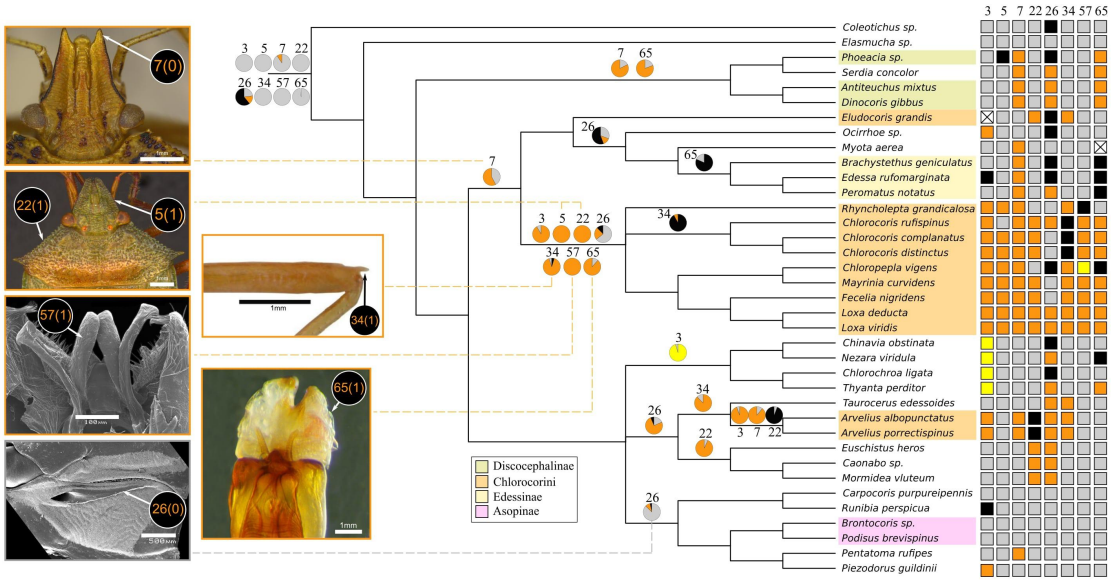

Figure 5. Maximum likelihood ancestral states of the key morphological characters of Chlorocorini reconstructed over the total evidence bayesian tree (converted to cladogram for visualization). Likelihood of ancestral states are shown as pie charts only in the nodes where evolutionary changes are likely to have happened (changes in terminal branches are omitted). Scoring for each taxon are exhibited as colored rectangles, where crossed rectangles are inapplicable states.