Genome size evolution in the beetle genus *Diabrotica*.

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Abstract

Diabrocite corn rootworms are one of the most economically significant pests of maize in the United States and Europe and an emerging model for insect-plant interactions. Genome sizes of several species in the genus *Diabrotica* were estimated using flow cytometry along with that of *Acalymma vittatum* as an outgroup. Within the *Diabrotica* subgroups fucata and virgifera, genome sizes ranged between 1.59 - 1.68 gigabase pairs (Gb) and between 2.31- 2.65 Gb, respectively, and the *Acalymma vittatum* genome size was around 1.69 Gb. This result indicated that a substantial increase in genome size occurred in the ancestor of the virgifera group. Further analysis of fucata group and virgifera group genome sequencing reads indicated that the genome size difference between the *Diabrotica* subgroups could be attributed to a higher content of

transposable elements, mostly miniature inverted-transposable elements (MITEs) and gypsy-like long terminal repeat (LTR) retroelements.

Introduction

The family Chrysomelidae is one of the largest families of phytophagous beetles (Order: Coleoptera), with nearly 40,000 species. A large number of species are important agricultural and forestry pests causing negative economic impacts (Reid, 1995; Nie et al., 2020). A subtribe of Chrysomelidae, Diabroticina includes important agricultural pests from the genera *Acalymma, Cerotoma,* and *Diabrotica* (Toepfer et al., 2009). Many species of the genus *Acalymma* are specialists on Cucurbitaceae, with *Acalymma vittatum*, the striped cucumber beetle, being one of the key pests of cucurbits in the northeastern United States (Lewis et al., 1990). The bean leaf beetle, *Cerotoma trifurcata,* is an important pest of leguminous crops such as peas and soybeans throughout the eastern USA (Koch et al., 2004). *Diabrotica*, the most diverse genus (Eben & Espinosa de los Monteros, 2013), is among the most destructive insect pests impacting US agriculture. *Diabrotica spp.* are divided into three groups: signifera, fucata, and virgifera, with the latter two containing recognized pest species (Krysan, 1986). The species in the fucata group are multivoltine and polyphagous, while species in the virgifera group are univoltine and oligophagous (Branson & Krysan, 1981; Krysan, 1982). *Diabrotica undecimpunctata* (southern corn rootworm) within the fucata group is a generalist feeder that feeds on several crops, including cucurbits, peanuts, and maize in the southern USA (Jackson et al., 2005). *Diabrotica virgifera virgifera* (western corn rootworm), *Diabrotica barberi* (northern corn rootworm), and *Diabrotica virgifera zeae*

(Mexican corn rootworm) from the virgifera group are specialist feeders and are pests of maize. *Diabrotica virgifera virgifera* is most abundant in the US Corn Belt but is found throughout much of the United States as well as parts of Canada and Mexico. *Diabrotica virgifera virgifera* and *D.barberi* are sympatric in the northern part of the US Corn Belt, while *D. v. zeae,* is sympatric with *D. v. virgifera* over part of their range in Texas, Arizona, and Mexico (Bragard et al., 2019). Diabroticina species show a wide range of host breadth, from monophagous to polyphagous. It has been found that the ancestors of Diabroticina were monophagous. The genus *Acalymma* has retained the ancestral monophagous habit, and the genus *Cerotoma* evolved oligophagy while the genus *Diabrotica* underwent an increase in diet breadth, shifting from monophagy to polyphagy. The fucata subgroup has retained the polyphagous characteristic, the virgifera subgroup, on the other hand, has become secondarily oligophagous (Eben & Espinosa de los Monteros, 2013).

As the name “corn rootworm” suggests, these insects cause substantial economic damage to maize. The western corn rootworm, *Diabrotica virgifera virgifera*, is considered one of the most destructive pests of maize throughout the US Corn Belt. More than $1 billion is spent on its control and lost yield every year (Sappington et al., 2006). The species has also been introduced into Europe and has become widespread because of a combination of transatlantic introductions and intra-continental movement (Ciosi et al., 2008; Miller et al., 2010; Ciosi et al., 2011). *Diabrotica barberi* is a serious maize pest but is less widespread than *D. v. virgifera* (Capinera, 2008). Although *D.undecimpunctata* is more widely distributed it is unable to survive the winter temperatures of the US Corn Belt, and it is considered an occasional pest of maize.

70 Diabroticina pests have repeatedly evolved behavioral and physiological
71 adaptations to several management strategies like chemical control, crop rotation, and
72 transgenic maize expressing *Bt* proteins (Levine & Oloumi-Sadeghi, 1991; Toepfer et
73 al., 2009; Miller et al., 2009; Eben & Espinosa de los Monteros, 2013; Devos et al.,
74 2013; Gassmann et al., 2014). *Diabrotica virgifera virgifera* evolved resistance to
75 cyclodiene insecticides during the late 1950s (Ball & Weekman, 1962; Sappington et al.,
76 2006; Miller et al., 2009), and later to carbamates, the organophosphate methyl-
77 parathion (Meinke et al., 1998; Miller et al., 2009), and most recently to pyrethroids
78 (Pereira et al., 2017; Souza et al., 2019; Souza et al., 2020). Both *D. v. virgifera* and *D.*
79 *barberi* have overcome crop rotation strategies through oviposition behavior (O'Neal et
80 al., 2002) and extended diapause (Krysan et al., 1984), respectively. Resistance to
81 transgenic maize expressing Cry3Bb1 and Cry34/35Ab1 proteins has also evolved in *D.*
82 *v. virgifera* and *D. barberi* (Gassmann et al., 2014; Calles-Torrez et al., 2019).
83 *Diabrotica virgifera virgifera* has also been shown to be capable of evolving resistance
84 to RNA interference (RNAi) in the laboratory (Khajuria et al., 2018).
85 Diabroticites have not only evolved resistance to control tactics but have also
86 evolved tolerance to plant-derived secondary metabolites used by plants to confer a
87 degree of protection against several insect herbivores. The benzoxazinoid DIMBOA
88 (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is one of the major secondary
89 metabolites produced by maize plants (Sasai et al., 2009). Cucurbitacins are another
90 group of bitter secondary metabolites found in the plants of the family Cucurbitaceae
91 (Rehm & Wessels, 1957). Studies have shown that *D. undecimpunctata* and *D. v.*
92 *virgifera* from the fucata and virgifera groups of *Diabrotica* can tolerate and sequester

93 the toxic secondary metabolites of plants such as cucurbitacins (Metcalf, 1986; Tallamy
94 et al., 1998) and DIMBOA (Robert et al., 2017) respectively. Although it has been found
95 that both these species can tolerate DIMBOA, they respond distinctly to this metabolite.
96 It was found *D. v. virgifera* gained significantly more dry weight when fed wild-type
97 plants for 10 days compared to being fed mutant plants, deficient for benzoxazinoid
98 biosynthesis. However, *D. undecimpunctata* performed equally well when fed on both
99 types of plants (Alouw & Miller, 2015). The enhanced performance of specialist *D. v.*
100 *virgifera* may be related to its ability to use DIMBOA as a signal to locate nutritious parts
101 of roots, while the generalist from the fucata group does not (Robert et al., 2012).
102 Further, RNA-Seq studies showed transcripts encoding for a CYP9-like cytochrome
103 P450 monooxygenase were expressed in *D. v. virgifera* larvae feeding on wild type
104 plants but not in larvae feeding on benzoxazinoid-deficient mutant plants (Miller & Zhao,
105 2015), suggesting a cytochrome P450 mediated adaptation to benzoxazinoids in *D. v.*
106 *virgifera*.
107 As several species in the *Diabrotica* genus can cause damage to crops and
108 adapt to control measures, there has been considerable interest in obtaining sequences
109 of their genomes and understanding genetic mechanisms for adaptations. Most
110 *Diabrotica* genetics and genomics research so far has been concentrated on *D. v.*
111 *virgifera* (Gray et al., 2009; Miller et al., 2010). The genome of *D. v. virgifera* is one of
112 the larger genomes among beetles and is estimated to be around 2.58 Gb (Coates et
113 al., 2012) whereas the average genome size for Coleoptera is 0.76Gb (Schoville et al.,
114 2018; Gregory, 2021). Sequencing the *D. v. virgifera* genome has posed a challenge.
115 Increased sizes of eukaryotic genomes are generally attributed to corresponding

116 increased numbers of repetitive DNA elements (Kidwell, 2002), where a large proportion
117 of repeats are composed of different transposable element (TE) sequences (Kojima,
118 2019). Eukaryotic transposons are divided into retroelements that propagate by an
119 RNA intermediate (class I) and DNA elements (class II) that mobilize by a "cut-and-
120 paste" mechanism (Finnegan, 1989; Wicker et al., 2007).

121 Studies also suggest that the *D. v. virgifera* genome contains a high proportion of
122 repetitive elements (Coates et al., 2012, 2014). The cadherin gene of *D. v. virgifera* is
123 approximately 13.3 fold larger than the *Tribolium castaneum* ortholog due to much
124 larger introns. The presence of numerous MITE-like elements within the cadherin gene
125 of *D. v. virgifera* indicates that the difference in the gene size is due to the insertion of
126 transposable elements in the *D. v. virgifera* introns (Coates et al., 2012). Class I BEL-
127 like long terminal repeat (LTR) retrotransposons have been also found in the *D. v.*
128 *virgifera* genome (Coates et al., 2014).

129 Although the genome size of *D. v. virgifera* has been reported, no genome size
130 data have been obtained for the other species in the genus *Diabrotica* and related
131 genera. Since the genome size of *D. v. virgifera* is relatively large, we hypothesized that
132 there has been a recent expansion in genome size in the lineage leading to it. We
133 tested this hypothesis by estimating and comparing the genome sizes of *D. v. virgifera*
134 with those of several *Diabrotica* species and an outgroup species, *A. vittatum.* As a high
135 proportion of repetitive elements were found in the cadherin gene of *D. v. virgifera,* we
136 further hypothesized that genome size expansion in the lineage leading to *D.v. virgifera*
137 was due to a general increase in repetitive elements. To test this hypothesis we looked

138 at the nature and quantity of repetitive elements in the virgifera group and compared it
139 with the fucata group.
140
141 **Materials and Methods**
142 **Sample Collection**
143 Specimens of *D. v. virgifera* and *A. vittatum* were collected from a maize field in Illinois
144 in 2017, while *D. barberi* were collected from Wisconsin by Tracy Schilder, Wisconsin
145 Department of Agriculture. Specimens of *D. v. zeae* were collected from Texas by
146 Thomas Sappington, US Department of Agriculture Agricultural Research Service, and
147 *D. balteata* were provided by Blair Siegfried and Heather McAuslane, University of
148 Florida, from a laboratory colony. *Diabrotica undecimpunctata* were obtained from Crop
149 Characteristics (Farmington, Minnesota, USA). Adult *Periplaneta americana,* which
150 were used as an external reference (Guo et al., 2015; He et al., 2016) for flow
151 cytometric measurement, were obtained from Carolina Biological Supply (Burlington,
152 North Carolina, USA). All samples were flash-frozen in liquid nitrogen and preserved at -
153 80°C.
154
155 **Sample Preparation**
156 Genome size estimates were generated for eight individuals from five species of
157 *Diabrotica* and one species of *Acalymma*. Preparations of nuclei were based on the
158 method of Hare and Johnston (2012). The heads of single individuals were
159 homogenized in 1ml of cold Galbraith buffer (4.26 g of $MgCl_2$, 8.84 g of sodium citrate,
160 4.2g of MOPS, 1ml of Triton-x, and 1mg of boiled ribonuclease A into 1 liter of ddH_20)

161 placed in a 7ml Kontes Dounce. The homogenate was filtered through a 20 µm nylon
162 mesh in a 1.5ml Eppendorf tube. Nuclei were stained with propidium iodide (PI) at 50
163 µg/ml, in the dark at 4°C for an hour. In addition to the test sample, the brain tissue of *P.*
164 *americana* was used as a standard (Hanrahan & Johnston, 2011). The brain tissue of *P.*
165 *americana* was dissected out, and the nuclear suspension was prepared and stained as
166 described above.
167
168 **Flow Cytometric Analysis**
169 Stained nuclei were analyzed using an Attune NxT Flow Cytometer (Thermo Fisher
170 Scientific, Waltham, Massachusetts). The propidium iodide-stained nuclei were excited
171 by exposing them to the 488 nm blue laser. Red fluorescence from the propidium iodide
172 was collected using a 610/20 emission filter in the YL2 detector channel. The calibration
173 of the flow cytometer was performed using a standard manufacturer's protocol before
174 use. The samples were inverted and mixed several times to resuspend and then loaded
175 onto the cytometer for analysis. During each sample run, the linearity of the
176 fluorescence measurement was confirmed by checking that the mean channel number
177 of the 4C nuclei (G2 phase) was double that of 2C nuclei (G1 phase). At least 1000
178 nuclear events were collected under each unknown and standard 2C peak. The nuclei
179 peak (PI fluorescence histogram) and coefficient of variation (CV) for each peak of
180 interest (sample and standard) were obtained using the gating function in the Attune
181 Software. The coefficient of variation (CV) was evaluated and confirmed to be less than
182 5 percent which is considered appropriate for accurate genome size estimates (Dolezel
183 et al., 2007; Tomaszewska et al., 2021). The known genome size of the standard

184 (3.41Gb, Harahan & Johnson, 2011) and the relative fluorescence obtained from the
185 sample and standard were then used to estimate the genome size using the following
186 formula:
187 Sample 1C DNA content = [(sample 2C mean peak position) / (standards 2C mean
188 peak position)] * standards 1C.
189 Genome size variations were analyzed using analysis of variance (ANOVA) and
190 Tukey's honest significant difference (HSD) post-hoc analyses using R statistical
191 software (version: 4.10) (*R Foundation for Statistical Computing, Vienna, Austria.*,
192 2020). Letters were assigned showing significance based on Tukey HSD post-hoc test
193 using R statistical software (version: 4.10) (R Core Team 2021).
194
195 **Sequencing Data**
196 Data from Illumina whole-genome shotgun sequencing were used to analyze the
197 repetitive DNA content of *D. barberi, D.undecimpunctata,* and *D. v. virgifera*. Adult *D.*
198 *barberi* (*n* = 71) and *D. undecimpunctata* (*n* = 50) were collected from maize fields near
199 Ames, Iowa, and Monmouth, Illinois, respectively. Each sample was pooled by species,
200 flash-frozen in liquid nitrogen and ground in liquid nitrogen, and then DNA extracted
201 from ~3.0 mg of tissue using the Qiagen DNeasy Blood and Tissue Extraction kit, with
202 modifications as described (Coates et al., 2014). Two micrograms of extracted DNA
203 was submitted to the Iowa State University DNA Facility (Ames, IA, USA) from which
204 ~500bp insert indexed sequencing libraries were generated using the Illumina TruSeq
205 v2 Library Construction Kit (Illumina, San Diego, CA). Single-end 100-bp reads were
206 generated from *D. barberi* and *D. undecimpunctata* libraries in separate lanes of an

207 Illumina HiSeq2500. Raw reads were submitted to the Biotechnology Information
208 (NCBI) Short Read Archive (SRA) under accessions SRR13363759 and SRR13364002
209 for *D. barberi* and *D. undecimpunctata*, respectively.
210 *Diabrotica virgifera virgifera* adult females of inbred line Ped12-6-A-3 were flash-frozen
211 prior to genomic DNA isolation. Briefly, whole beetles were homogenized in an SDS-
212 based cell lysis solution followed by overnight incubation with Proteinase K at 55°C.
213 Cellular debris was pelleted and RNA was digested with RNaseA. The homogenate was
214 mixed with a high-salt solution and incubated overnight at 4°C. The DNA in the
215 supernatant was precipitated overnight with ethanol at -20°C. DNA was quantified on an
216 Invitrogen Qubit. A paired-end short-insert genomic DNA library was prepared at the
217 Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign
218 using an Illumina TruSeq DNAseq Sample Prep kit. Reads were sequenced to 100bp
219 with the Illumina TruSeq SBS sequencing kit version 3 on an Illumina HiSeq 2000
220 instrument using Casava 1.8 for basecalling.
221 Sequencing generated 90 million single-end reads of 100bp for *D. barberi,* 118 million
222 single-end reads of 100bp for *D.undecimpunctata*, and 116 million 100-bp paired-end
223 reads for *D. v. virgifera*.
224
225 **Annotation and Quantification of Repeat Content**
226 Raw reads were quality-filtered using fastp software (version 0.20.1) with a minimum 20
227 average Phred score. Reads mapping to mitochondrial genome sequences of
228 *Diabrotica* species available through the NCBI website (KF658070.1, KF669870.1) were
229 identified (minimap2 v2.17) and filtered out as implemented in the SSRG workflow

230 (Pombert, 2021). Repetitive elements in the genomes of *D.undecimpunctata, D. barberi,*
231 and *D. v. virgifera* were assembled and quantified using dnaPipeTE v1.3 (Goubert et al.,
232 2015) and annotated using the DeepTE tool (Yan et al., 2020). To quantify the
233 proportion of TEs, dnaPipeTE uses samples of sequence reads instead of genome
234 assemblies, making this pipeline (dnaPipeTE) applicable for genomes with lower
235 sequencing depth. The pipeline performed assembly of repetitive reads into contigs
236 from low coverage sampling of raw reads using Trinity (Grabherr et al., 2011) and
237 annotated them using RepeatMasker (Smit et al., 1996-2004) with built-in Repbase
238 libraries (Bao et al., 2015, version 2017-01-27). Quantification was done by mapping a
239 random sample of reads onto the assembled repeats. The parameters set as the
240 benchmark for repeat content analysis for genomes greater than 500Mb (Goubert et al.,
241 2015), including the coverage parameter, were used to run dnaPipeTE. The pipeline
242 was run for all three species using 0.1x coverage. Additionally, 0.1x coverage was
243 chosen based on the high N50 metric and plateauing point of transposable elements i.e,
244 increasing the coverage beyond 0.1x only marginally increased the proportion of
245 transposable elements for all three species. The dnaPipeTE pipeline does not annotate
246 novel repeats that do not match an entry in the included Repbase library. A high
247 proportion of repeats from each of the three beetle species were not annotated by
248 dnaPipeTE. DeepTE, a deep learning method based on convolutional neural networks,
249 was used to classify and annotate the unknown TEs. DeepTE uses eight trained models
250 to classify TEs into superfamilies and orders. All the TE contigs assembled by
251 dnaPipeTE were analyzed using DeepTE, whether or not they had been previously
252 classified by dnaPipeTE. Combining the results of the assembly and quantification by

253 dnaPipeTE with the classification results from DeepTE allowed the abundance of repeat
254 families in the genomes of all three species to be determined.

255 For comparison with the dnaPipeTE *de-novo* assembly of repetitive elements, the
256 percentage of repetitive elements in the *D. v. virgifera* genome assembly (NCBI RefSeq
257 accession GCF_003013835.1) was analyzed with RepeatModeler version 2.0.1 (Flynn
258 et al., 2019). Repeatmodeler is a *de-novo* transposable element identification package
259 that uses three repeat finding programs (RECON, RepeatScout, and
260 LtrHarvest/Ltr_retriever) to discover repetitive DNA sequences in the genome. These
261 repetitive DNA sequences were annotated by repeatClassifier based on the similarity to
262 RepBase and Dfam databases. The annotated library produced was used as input to
263 RepeatMasker to detect and mask repeats in the genome. Default parameters were
264 used to run RepeatModeler.
265

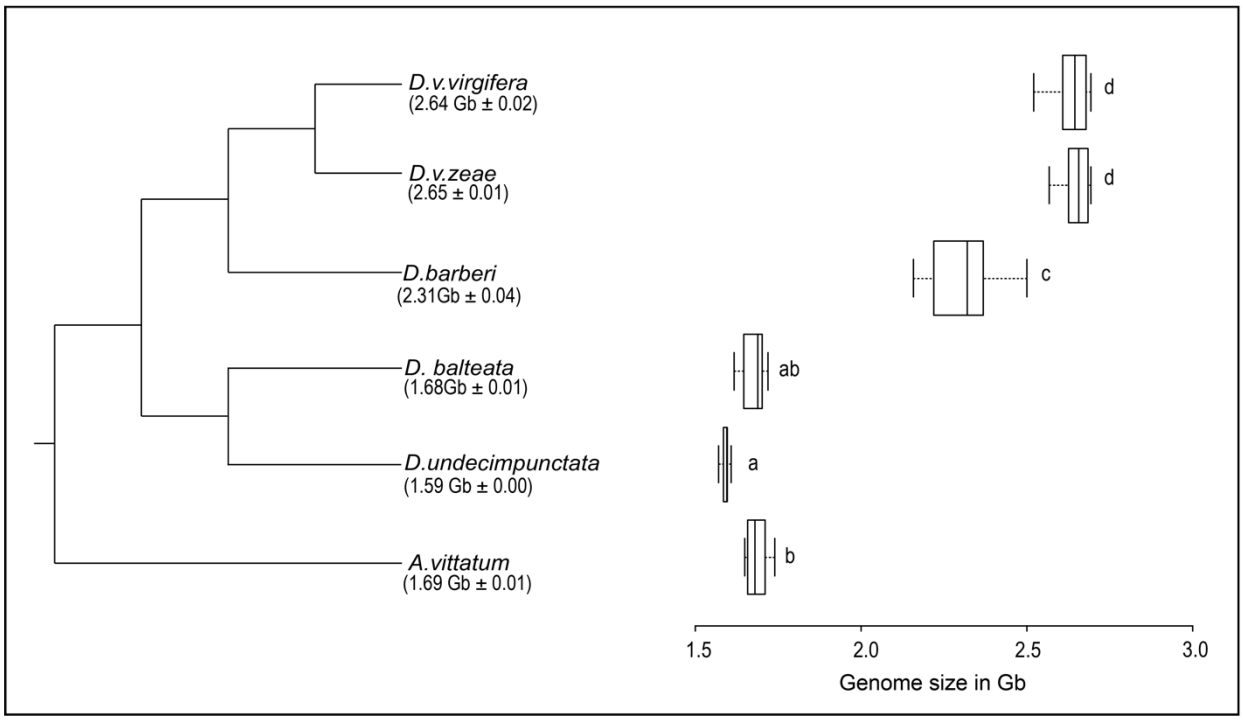
266 **Results**

267 **Genome size from flow cytometry:**

268 The genome sizes of *D. v. zeae*, *D. v. virgifera,* and *D. barberi* from the virgifera group
269 were estimated to be 2.65Gb ± 0.01, 2.64 Gb ± 0.02, and 2.31Gb ± 0.04, respectively.
270 The genomes of *D. balteata* and *D.undecimpunctata* from the fucata group were
271 estimated at 1.68Gb ± 0.01 and 1.59 Gb ± 0.00. The outgroup species *A. vittatum*
272 genome size is estimated to be 1.69 Gb ± 0.01. An analysis of variance (ANOVA)
273 showed a significant difference ($p < 2 \times 10^{-16}$) in the genome sizes of the species
274 under study. A subsequent Tukey HSD test showed that there were no significant
275 differences in genome size between *D. v. virgifera* and *D. v. zeae*, between *D. balteata*

276 and *D.undecimpunctata,* or between *D. balteata* and *A. vittatum*. The estimated genome

277 size for each species with their phylogenetic relationships is shown in Figure I.



278

279 Figure I: Genome size evolution within the genus *Diabrotica.* Phylogeny of *Diabrotica* and outgroup

280 *Acalymma vittatum* is based on Eben & Espinosa de los Monteros, 2013. Letters a - d indicate groups

281 with no significant difference in mean 1C-value (Tukey HSD; α = 0.05).

282

283 **Repeat Content analysis:**

284 The repeatomes of *D.v.virgifera, D. barberi,* and *D.undecimpunctata* comprised 72.5%,

285 70.4%, and 52.7% of their genomes, respectively. The repeat content obtained via

286 Repeat Modeler for the draft *D. v. virgifera* genome was 57.4%. As the assembly of the

287 *D.v. virgifera* genome was based on short-reads, TEs were expected to be under-

288 represented because of the difficulty of assembling individual copies. TE-rich large

289 genomes are difficult to assemble and often end up with high levels of fragmentation

290 around repetitive regions leading to underestimation of TE content (Green, 2002).

291 To further investigate the classes of repeat families that contributed to the genome size
292 variation between the two groups of *Diabrotica*, the DeepTE annotations were coupled
293 with the dnaPipeTE abundance quantification to estimate the abundance of different
294 repeat elements in the genomes of *D. v. virgifera, D.barberi,* and *D.undecimpunctata*.
295 The TEs that accounted for most of the difference in the genome size of the two groups
296 were annotated as class II DNA Tc1-mariner Miniature-repeat Transposable Elements
297 (MITEs) (ClassII_DNA_TcMar_MITE) and class II DNA hAT MITE
298 (ClassII_DNA_hAT_MITE) of transposable elements and class I long terminal repeat
299 (LTR) Gypsy (ClassI_LTR_Gypsy) transposable elements (Figure II). The *D. v. virgifera*
300 genome contained a large amount of ClassII_DNA_TcMar_MITE(0.62 Gb),
301 ClassII_DNA_hAT_MITE (0.24 Gb), and ClassI_LTR_Gypsy(0.31 Gb). Similarly, *D.*
302 *barberi* also had a high amount of ClassII_DNA_TcMar_MITE(0.53 Gb),
303 ClassII_DNA_hAT_MITE (0.19 Gb), and ClassI_LTR_Gypsy(0.26 Gb). The *D.*
304 *undecimpunctata* had a lower amount of ClassII_DNA_TcMar_MITE(0.16 Gb),
305 ClassII_DNA_hAT_MITE (0.06 Gb), and ClassI_LTR_Gypsy(0.17 Gb). Transposable
306 elements from other repeat families such as nLTRS, helitrons, and others from class I
307 and class II DNA elements were not as prominent as those mentioned above. Single-
308 low copy sequences representing the non-repetitive portion of the genomes of *D. v.*
309 *virgifera*, *D.barberi*, and *D.undecimpunctata* totaled 0.73 Gb, 0.68 Gb, and 0.75 Gb,
310 respectively.

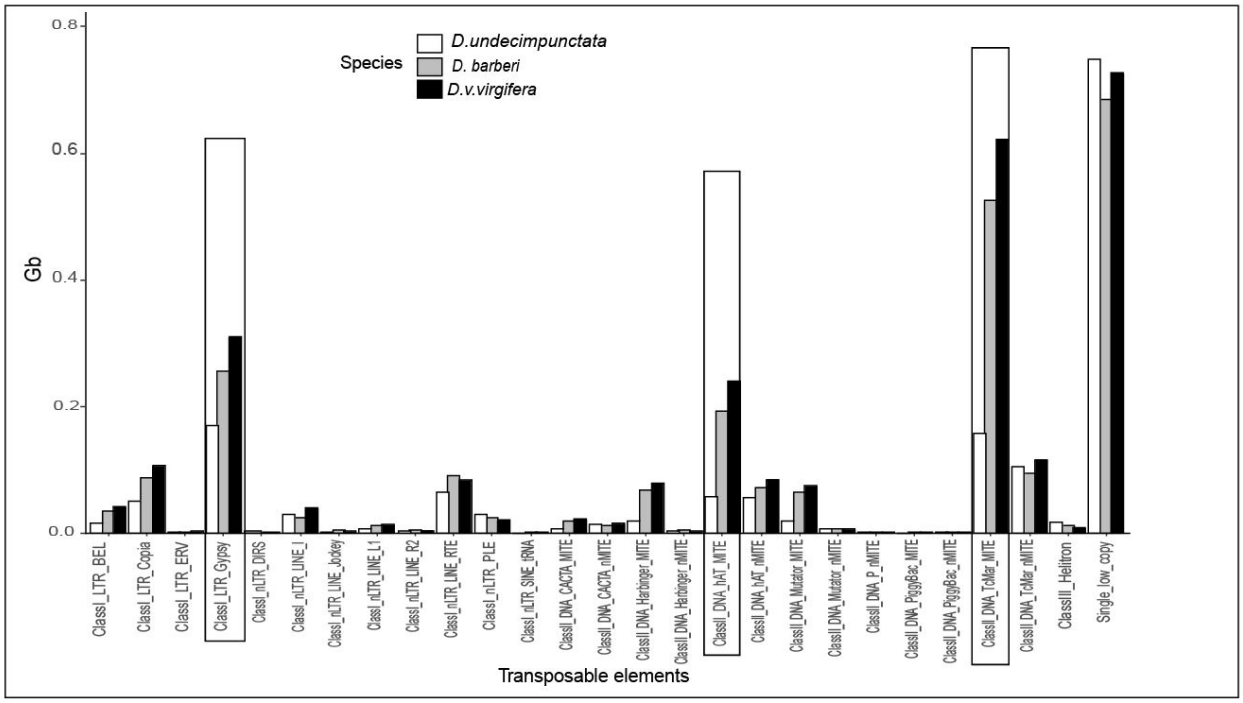


Figure II: Quantities of transposable elements present in the genomes of three species of *Diabrotica.* The boxed transposable elements are those with high contributions to the genome size variation between the groups of fucata and virgifera.

Discussion:

Diabrotica is the most diverse genus within the subtribe Diabroticina and includes 354 species native to America. Only species from the fucata and virgifera groups occur in the United States (Krysan, 1986), while species from the signifera group are endemic to South America. The economically significant pest species in this genus either belong to the virgifera group or to the fucata group, justifying the need to study them comprehensively. The signifera group species are not of economic importance, and their biology is also mostly unknown (Clark et al., 2001) and so the group is understudied. The expansion and adaptability of the pest species generates a sense of urgency to study them.

326 Our results demonstrated that the genome size of virgifera group species are
327 approximately 1Gb larger than that of the fucata group and *Acalymma* species. The
328 genome size for *D. v. virgifera* obtained in this study and in a previous study (Coates et
329 al., 2012) are consistent. Our genome size results, when coupled with the species'
330 phylogenetic relationship, indicated that an expansion in genome size occurred in the
331 common ancestor of the virgifera group leading to *D. barberi, D. v. virgifera,* and *D. v.*
332 *zeae*. There was also a significant difference in the genome sizes of *D. barberi* and *D.*
333 *virgifera subsp.* suggesting a possible further expansion of the genome size in the
334 common ancestor of *D. v. virgifera* and *D. v. zeae*.
335 The estimated divergence time between *Acalymma* and *Diabrotica* species and
336 between the *Diabrotica* species group was dated at 45 million years ago (Mya) and 31
337 Mya respectively. Another radiation within the Diabrotica genus took place around 17
338 Mya resulting in virgifera group (Eben & Espinosa de los Monteros, 2013). Repeat
339 elements of species from fucata and virgifera were studied to understand the basis of
340 genome size expansion in *Diabrotica.* The genomes of the virgifera and fucata group of
341 *Diabrotica* species contained many common TEs, but the abundance of three TE
342 families differed substantially and accounted for much of the difference in genome size
343 between the two groups and also within the virgifera group between *D.barberi* and *D.*
344 *virgifera subsp*. The MITE-like Tc1-mariner and hAT elements and LTR Gypsy
345 retroelements were more abundant in the virgifera group. Miniature-repeat
346 Transposable Elements (MITEs) are short AT-rich (<0.5kb) derivatives of DNA elements
347 whose internal sequence lacks an open reading frame (Lu et al., 2012), contain
348 conserved terminal inverted repeats, are flanked by target site duplications, and are

349 closely associated with euchromatic genes (Kuang et al., 2009). Gypsy elements are
350 one of the most abundant classes of the long terminal repeat (LTR)-retrotransposons
351 superfamily with large numbers of copies found in almost all the plants, animals, and
352 fungi tested (Thomas-Bulle et al., 2018). Generally, class I transposable elements have
353 been reported to be in high abundance in insect genomes, such as *Tribolium*
354 *castaneum* (Wang et al., 2008), *Drosophila* (Clark et al., 2007)*, Bombyx mori* (Osanai-
355 Futahashi et al., 2008) in comparison to class II elements. However, in *Diabrotica*, we
356 discovered an abundance of class II elements and, to some extent, class I elements.

357 Genome size varies enormously among eukaryote species (Hidalgo et al., 2017), and
358 among beetles in the Family Chrysomelidae (Hanrahan & Johnston, 2011; Petitpierre et
359 al., 1993). There are studies showing a positive correlation between genome size and
360 phenotypic traits such as body size in groups of invertebrates, including aphids (Finston
361 et al., 1995) and flies (Ferrari & Rai, 1989). Equally, there are studies showing negative
362 correlations between phenotypic traits and genome sizes, such as the relation between
363 body size and genome size for the *Pimelia* and *Phylan* genera in the beetle family
364 Tenebrionidae (Palmer et al., 2003; Palmer & Petitpierre, 1996). Cases of negative
365 correlation of genome size with pupal development have been reported in ladybird
366 beetles and with the overall development rate in *Tribolium* (Carreras et al., 1991;
367 Gregory et al., 2003). It has been previously hypothesized that genome size negatively
368 correlates with the host range in insect herbivores (Zhang et al., 2019). However, there
369 are also studies showing that genome size may be positively correlated with the host
370 range in insect herbivores (Matsubayashi & Ohshima, 2015; Calatayud et al., 2016).

371 This contradiction in results, along with our data suggests that the genome size may not
372 be related directly to the host range.

373 Comparative genomic studies in insects have revealed that repeat elements can make
374 large contributions to genome size variation. Variation in TE abundance can be seen
375 both within and among species (Lynch, 2007). Honeybees, with a genome size of
376 230Mb, show very few repeat elements, representing a case of TE extinction
377 (Weinstock et al., 2006). Similarly, the small genome of *Belgica antarctica,* the Antarctic
378 midge, (99 Mb) is also due to the reduction of repeats in the genome (Kelley et al.,
379 2014). There are also cases of TE proliferations, such as in *Locusta migratoria,* that led
380 to its large genome size of 6.5 Gb (Wang et al., 2014). Cases of increase in genome
381 size as a consequence of transposable elements have also been reported in wood
382 white (Leptidea) butterflies and North American fireflies (Lampyridae) (Talla et al., 2017;
383 Lower et al., 2017). This further suggests that genome size in insects is fairly plastic and
384 largely driven by the loss and gain of transposable elements.

385 Host genomes have employed several mechanisms to suppress transposable
386 element expression and mobility (Bourque et al., 2018) which includes epigenetic
387 silencing either through histone modifications or DNA methylation, targeted
388 mutagenesis, small RNA interference, as well as sequence-specific repressors such as
389 the recently profiled KRAB zinc-finger proteins (Fouché et al., 2020; Maupetit-Mehouas
390 & Vaury, 2020). At the same time, some transposable elements have evolved regulatory
391 sequences controlling their own copy number to autonomously replicate in the genome
392 (Lohe & Hartl, 1996; Saha et al., 2015). TE de-repression is triggered by environmental
393 stimuli, in particular stress (Bundo et al., 2014; Fouché et al., 2020; Voronova et al.,

394 2014) impacting transcription levels and increasing transpositional activity (Dubin et al.,
395 2018). Additionally, there are other factors influencing TE mobilization for example
396 demethylation and the removal of repressive histone marks during epigenetic
397 reprogramming stages (Russell & LaMarre, 2018)

398 Transposable elements are a major component of many eukaryotic genomes and
399 have been associated with several mechanisms shaping eukaryotic genome function,
400 structure, and evolution (Charlesworth & Charlesworth, 1983; Chuong et al., 2017).
401 These transposons are capable of changing position within the genome, leading to
402 mutations by inserting themselves into functional regions and causing change by either
403 modifying or eliminating gene expression (Feschotte, 2008; Oliver & Greene, 2009). TE
404 integration and excision can introduce novel variation at the individual and population-
405 scale (McClintock, 1950; Wendel & Wessler, 2000) introducing phenotypic modifications
406 through changes in epigenetic mechanisms and providing genetic motifs (Jiang et al.,
407 2004; Kapitonov & Jurka, 2007; Stuart et al., 2016; Wessler et al., 1995). They may also
408 lead to genomic rearrangement (Maumus et al., 2015; Mat Razali et al., 2019). In
409 particular, MITES and LTR retrotransposon have played an essential role in shaping
410 gene structure and altering gene function (Wessler et al., 1995).

411 Initially, MITEs were found as key components of plant genomes, as they are
412 frequently associated with genes with high copy numbers indicating a possible role in
413 gene expression and genome evolution (Santiago et al., 2002; Oki et al., 2008). They
414 are also found in animals including mosquitoes, *Drosophila*, fish, and humans (Deprá et
415 al., 2012). Similar to MITES, LTR retrotransposons were also first discovered in plants.
416 They are usually located largely in intergenic regions and are often the single largest

417 component of plant genomes (Feschotte et al., 2002; Kumar & Bennetzen, 1999).
418 Previous studies have also revealed that both MITES and LTR retrotransposons can
419 regulate downstream gene expression by inserting into promoter regions (Wang et al.,
420 2017; Butelli et al., 2012; Liu et al., 2019).

421 Mostly, TE insertions are presumed to be deleterious or neutral, but some have
422 been shown to be selectively advantageous. There are several studies showing that TE-
423 mediated insertions have led to insecticide resistance. In pink bollworm *Pectinophora*
424 *gossypiella*, a major pest of cotton (Rostant et al., 2012), several independent TE
425 insertions in the *PgCad1* gene conferred resistance to *Bt* Cry1Ac toxin (Fabrick et al.,
426 2011; Wang et al., 2019). Cases of resistance to *Bacillus thuringiensis* (Bt) toxins have
427 also been reported in *Heliothis virescens* which is caused by disruption of a cadherin-
428 superfamily gene by TE insertion (Gahan, 2001). TE insertions in xenobiotic metabolism
429 related genes such as those encoding cytochrome P450 monooxygenases and
430 glutathione S-transferases in *Helicoverpa armigera* are the cause of resistance to
431 insecticides (Klai et al., 2020). Another example demonstrating that TEs can produce
432 adaptive mutations has been reported in *D. melanogaster* (Rostant et al., 2012; Gilbert
433 et al., 2021). An increased resistance to dichlorodiphenyltrichloroethane (DDT) in *D.*
434 *melanogaster* has been reported due to Cyp6g1 upregulation caused by insertion of the
435 Accord transposon in the 5′ regulatory region of the Cyp6g1 gene (Chung et al., 2007).
436 Similarly, TE insertion, which truncates the CHKov1 gene in *D. melanogaster*, confers
437 resistance towards organophosphate (Aminetzach, 2005).

438 The role played by TEs, if any, in Diabroticina evolution, both long term such as
439 speciation and host plant shifts, and in recent evolution, such as their adaptation to pest

management, is unknown. However, the ongoing effort of the United States Department of Agriculture Agricultural Research Service (USDA-ARS) Ag100Pest initiative to sequence other species of *Diabrotica* may help us understand and answer these questions.

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