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Combining genetic crosses and pool targeted DNA-seq for untangling genomic variations associated with resistance to multiple insecticides in the dengue vector *Aedes aegypti*

Short title: Genomic variations associated with insecticide resistance in the dengue vector Aedes aegypti

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Abstract

In addition to combating vector-borne diseases, studying the adaptation of mosquitoes to insecticides provides a remarkable example of evolution-in-action driving the selection of complex phenotypes. Indeed, most resistant mosquito populations show multi-resistance phenotypes as a consequence of the variety of insecticides employed and of the complexity of selected resistance mechanisms. Such complexity makes challenging the identification of alleles conferring resistance to specific insecticides and prevents the development of molecular assays to track them in the field. Here we showed that combining simple genetic crosses with pool targeted DNA-seq can enhance the specificity of resistance allele's detection while maintaining experimental work and sequencing effort at reasonable levels. A multi-resistant population of the mosquito *Aedes aegypti* was exposed to three distinct insecticides (deltamethrin, bendiocarb and fenitrothion) and survivors to each insecticide were crossed with a susceptible strain to generate 3 distinct lines. F2 individuals from each line were then segregated with 2 insecticide doses. Bioassays supported the improved segregation of resistance alleles between lines. Hundreds of genes covering all detoxifying enzymes and insecticide targets together with more than 7,000 intergenic regions equally spread over mosquito genome were sequenced from pools of F0 and F2 individuals unexposed or surviving insecticide. Differential coverage analysis identified 39 detoxification enzymes showing an increased gene copy number in association with resistance. Combining an allele frequency filtering approach with a Bayesian F_{ST}-based genome scan identified multiple genomic regions showing strong selection signatures together with 50 non-synonymous variations associated with resistance. This study provides a simple and cost-effective approach to improve the segregation of resistant alleles in multi-resistant populations while reducing false positives frequently arising when comparing populations showing divergent genetic backgrounds. The identification of these insecticide resistance markers paves the way for the design of novel DNA-based resistance tracking assays.

Key words: Insecticide resistance, Mosquito, *Aedes aegypti*, Copy Number Variations, Polymorphism, Complex phenotype, Detoxification enzymes, Cytochromes P450.

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Author summary

In addition to combating vector-borne diseases, understanding how mosquitoes adapt to insecticides provides a remarkable example of evolution-in-action. However, the variety of insecticides used and the complexity of adaptive mechanisms make it difficult to identify the genetic changes conferring resistance to each insecticide. Here we combined simple controlled crosses with mass DNA sequencing for enhancing the specificity of resistance gene detection. A multi-resistant mosquito population was exposed to three distinct insecticides and survivors were crossed with a susceptible strain to generate 3 distinct mosquito lines. Individuals from the second generation of each line were then segregated based on their resistance to each insecticide. Bioassays supported the improved segregation of genetic resistance markers between lines. Hundreds of genes potentially involved in resistance together with thousands non-genic regions equally spread over mosquito genome were sequenced from individuals from each line. Genomic analyses identified detoxification enzymes showing an increased gene copy number in association with resistance and multiple genomic regions showing strong selection signatures and carrying point mutations associated with resistance. Such approach improves the specificity of resistance gene detection in field mosquito populations resisting to multiple insecticides and paves the way for the design of novel DNA-based resistance tracking tools.

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48 # Introduction

49 Natural populations experience a variety of selective pressures, leading to the accumulation
50 of locally adaptive features and the expression of complex phenotypes [1]. Environmental
51 changes driven by man-made disturbances can alter the course of selection, by inducing
52 novel, particularly strong and sometimes unpredictable selective pressures. Understanding
53 how natural populations respond to rapid environmental changes has become a major goal,
54 and an increasing number of studies reported adaptive changes on very short timescales [2-
55 4]. Resistance of insects to insecticides is a key example of rapid evolution under novel and
56 strong selective pressures associated with human activities. This adaptive phenotype has
57 evolved quickly and independently in a large number of taxa [5]. However, natural resistant
58 populations often exhibit complex resistance phenotypes as a consequence of the variety of
59 insecticides used, the intensity of the selection pressures, and the selection of mechanisms
60 conferring resistance to multiple insecticides, making the identification of resistance alleles
61 challenging [6, 7]. Besides contributing to the understanding of rapid adaptation and the
62 origins of complex traits, deciphering the complexity of insecticide resistance mechanisms
63 could help improving risk assessments and management strategies [8].

64 Among taxa of serious economic and medical importance, mosquitoes are vectors of
65 numerous human viruses and pathogens representing a major threat for public health
66 worldwide [9]. Among them, *Aedes aegypti* is of particular importance because of its wide
67 distribution [10] and its capacity to transmit several major arboviral diseases including Yellow
68 Fever, Dengue, Zika fever and Chikungunya fever. Although efforts are invested in
69 developing novel vaccines and strategies to prevent arbovirus transmission, the use of
70 chemical insecticides remains the cornerstone of arboviral diseases control. However, as for
71 malaria vectors, decades of chemical treatments have led to the selection and spread of
72 resistance in this mosquito species. Insecticide resistance is now widespread in *Ae. aegypti*
73 and affects all insecticide families used in public health [11], often leading to reduced vector
74 control efficacy [12-14]. Although attempts are made to develop alternative arbovirus control
75 strategies [15] their large scale implementation will require decades. Until this, characterizing
76 molecular mechanisms underlying resistance is crucial for tracking down resistance alleles
77 and improving resistance management strategies [16].

78 Resistance of mosquitoes to chemical insecticides can be the consequence of various
79 mechanisms, such as non-synonymous mutations affecting the protein targeted by
80 insecticides, a lower insecticide penetration, its sequestration, or its biodegradation often
81 called metabolic resistance [6, 17]. In *Ae. aegypti*, resistance to pyrethroids, the main
82 insecticide family used against mosquitoes, is mainly the consequence of target-site

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83 mutations affecting the voltage-gated sodium channel targeted by these insecticides (Knock
84 Down Resistance '*kdr*' mutations) and metabolic mechanisms [11, 18]. Several *kdr* mutations
85 have been identified in this species and the causal association between the V410L, S989P,
86 V1016G/I and F1534C mutations and pyrethroid resistance has been confirmed [19-23].
87 Most of these mutations can be genotyped on individual mosquitoes by PCR-based assays,
88 providing essential allele frequency data for resistance management. Conversely, metabolic
89 resistance is far less understood in *Ae. aegypti* although this type of resistance is frequent
90 and often accounts for a significant part of the resistance phenotype [6]. Such resistance
91 mechanism is caused by an increased activity of detoxification enzymes. These
92 detoxification enzymes include cytochrome P450 monooxygenases (P450s or *CYPs* for
93 genes), carboxy/cholinesterases (CCEs), glutathione S-transferases (GSTs) and UDP-
94 glycosyl-transferases (UDPGTs) although other families can be involved [17, 18, 24]. Their
95 high diversity (~ 300 genes in *Ae. aegypti*) and the complexity of biodegradation pathways
96 make challenging the identification of those conferring resistance to a specific insecticide.
97 Theoretically, metabolic resistance can be the consequence of an increased expression of
98 one or multiple detoxification enzymes metabolizing the insecticide and/or the selection of
99 variants showing a higher insecticide metabolism rate due to conformal modifications. As
100 over expression is frequently associated with over transcription, most candidate genes were
101 identified based on their differential transcription in resistant populations as compared to
102 susceptible counterparts using transcriptomics [11, 18, 24, 25]. Although these approaches
103 identified several detoxification enzymes involved in insecticide biodegradation, they mostly
104 failed to pinpoint the underlying genomic changes, thus impairing the high-throughput
105 tracking of metabolic resistance alleles in natural populations. The application of powerful
106 genomic tools has improved the understanding of the genetic bases of metabolic resistance
107 in *Ae. aegypti*. Using deep targeted DNA sequencing (targeted DNA-seq) Faucon et al. [26]
108 identified genomic variations associated with resistance to the pyrethroid deltamethrin in
109 multiple populations sampled from different continents. This study identified several
110 detoxification enzymes affected by Copy Number Variations (CNV) and non-synonymous
111 variations in association with insecticide resistance. Cross-comparing these genomic data
112 with transcriptomic data obtained from RNA-seq confirmed the central role of CNV in the
113 over-expression of detoxification enzymes associated with resistance in this species [27].
114 However, this study used natural resistant populations displaying multi-resistance
115 phenotypes, thus not allowing to properly discriminating between alleles specifically
116 associated with resistance to the insecticide in question and those associated with resistance
117 to other insecticides. Furthermore, this approach did not allow breaking up the genetic
118 linkages between the genomic variations identified, thus potentially leading to false positives.

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119 In this context, the present study aimed at better understanding the origin of complex
120 insecticide resistance phenotypes in the mosquito *Ae. aegypti*. More precisely, we combined
121 genetic crosses and targeted DNA-seq in an attempt to identify genomic variations
122 specifically associated with resistance to distinct insecticides in a multi-resistant *Ae. aegypti*
123 population. After exposure to three insecticides of distinct chemical families (the pyrethroid
124 deltamethrin, the organophosphate fenitrothion and the carbamate bendiocarb), survivors to
125 each insecticide were crossed with a susceptible strain to generate three F2 lines. Each F2
126 line was then phenotyped with two increasing doses of its respective insecticide and
127 survivors were used to identify CNV and polymorphism variations associated with resistance
128 in hundreds of target genes including all detoxification enzymes and insecticide target
129 proteins. In addition, the capture of thousands of intergenic regions regularly distributed over
130 mosquito genome also allowed crossing up these data with a genome-wide screening of
131 selection signatures associated with resistance. Overall, this study contributes to improve our
132 understanding of the complex genomic bases of metabolic resistance to insecticides and
133 paves the way for the design of novel insecticide resistance tracking tools in this major
134 arbovirus vector.

135

136 Results

137 Insecticide resistance levels

138 The population used in this study consisted in a composite population representative of
139 multiple natural *Ae. aegypti* populations collected from French Guiana (see methods).
140 Bioassays performed on the initial composite population (F0 Guy-R) confirmed its high
141 resistance to the pyrethroid insecticide deltamethrin with resistance ratio (RR_{50}) over 316-fold
142 as compared to the susceptible strain Bora-Bora (Fig. 1A). This population also showed
143 moderate resistance to the carbamate bendiocarb and the organophosphate fenitrothion with
144 RR_{50} of 14-fold and 3-fold respectively. As expected, resistance to each insecticide
145 decreased after crossing F0 survivors to each insecticide with the susceptible strain with F1
146 resistance ratios decreasing to 25-fold, 7-fold and 2-fold for deltamethrin, bendiocarb and
147 fenitrothion respectively. Deltamethrin resistance was even lower in F2 (10-fold) while
148 fenitrothion resistance remains low (1.8-fold) and bendiocarb resistance slightly increased to
149 10-fold. Assessing the cross resistance of each line to all insecticides confirmed the partial
150 segregation of resistance alleles after controlled crosses (Fig. 1B). Although F2 individuals
151 from each line showed a higher survival when exposed to its respective insecticide, this trend
152 was only significant for deltamethrin in link with the lower resistance to other insecticides.

153

154 Target-site resistance mutations

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155 Assessing kdr mutations frequencies from targeted DNA-seq reads data confirmed the high
156 frequency of the three kdr mutations V410L, V1016I and F1534C in F0 Guy-R composite
157 population corroborating its high deltamethrin resistance level (Fig. 2). Exposing F0 Guy-R
158 individuals to the LD_{80} of each insecticide did not segregate these mutations in survivors. The
159 segregation of kdr mutations became more evident in F2 individuals with higher allele
160 frequencies observed in F2 individuals of the Delt line surviving to high dose of deltamethrin.
161 As expected because genetically constrained by two successive mutation events in *Ae.*
162 *aegypti* [28], the acetylcholinesterase G119S mutation conferring resistance to
163 organophosphates and carbamates in other species was not detected for DNA-seq reads
164 data. Validation of kdr mutation frequencies on individual mosquitoes by qPCR confirmed the
165 robustness of allele frequencies estimated from DNA-seq although moderate discrepancies
166 were observed when the number of genotyped mosquitoes was low (S1 Fig.).
167

168 **Gene copy number variations**

169 Over 49.5 % of sequenced reads were successfully mapped to AaegL5 exome allowing the
170 detection of 1,317 exonic regions (719 distinct genes) showing a minimum length of 45 bp
171 and a coverage between 30 and 800 reads/bp in all conditions (median = 94.1 reads/bp).
172 Filtering genes based on their expected CNV profiles across F0 and F2 conditions in each
173 line (see methods for filtering conditions) allowed identifying 39 detoxification genes affected
174 by CNV in association with insecticide resistance (Fig. 3 and S1 Table). Although the
175 resistance level of the Delt line was high, more CNV were detected in the two other lines
176 likely due to the contribution of kdr mutations in the deltamethrin resistance phenotype. This
177 trend was also observed for CNV intensity as most genes identified in the Delt line showed a
178 lower CNV increase in F2 individuals surviving high dose of insecticide as compared to those
179 identified in the Bend and Feni lines.

180 Among genes affected by CNV, 11 were associated with resistance to multiple insecticides
181 including the P450s *CYP6P12* and *CYP304B2* and the CCE *AAEL010592* being associated
182 with resistance to all insecticides. Genes affected by CNV in association with deltamethrin
183 resistance included 6 P450s, 4 GSTs, 1 alcohol dehydrogenase and 1 CCE. Among them, 2
184 CYP6 genes belonging to a cluster of P450s in chromosome 1 and 2 CYP9Js belonging to a
185 large cluster of P450s on chromosome 3 were previously identified as affected by CNV
186 associated with deltamethrin resistance [26]. Genes affected by CNV in association with
187 bendiocarb resistance included 15 P450s, 3 GSTs and 2 CCEs. P450s included all genes of
188 the CYP6 cluster located on chromosome 1 and 4 genes of the large CYP9J cluster located
189 on chromosome 3 but also several genes from the CYP4, CYP6 and CYP304 families. The
190 CYP6-like *AAEL009018* located on chromosome 1 was specifically associated with

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191 bendiocarb resistance with a marked CNV increase in both F0 and F2 survivors. Genes
192 affected by CNV in association with fenitrothion resistance included 11 P450s, 3 GSTs, 3
193 glycosyl-transferases (UDPGTs), 2 CCEs and 1 sulfotransferase. Genes specifically
194 associated with fenitrothion resistance included the sulfotransferase *AAEL004557* and the
195 P450 *CYP304B3* on chromosome 1 and the UDPGT *AAEL005468* located at the end of
196 chromosome 3, but most genes were located at the end of chromosome 2 (*CYP6N17*,
197 *CYP6Z8*, *CYP6M5*, *GSTX2*, UDPGT *AAEL000687*).

198

199 **Selection imprints and polymorphisms**

200 Over 85% of sequenced reads were successfully mapped to AaegL5 genome allowing the
201 detection of more than 40,000 variations. Among them, 24,714 were polymorphic across
202 conditions and passed quality and coverage filters (S2 Table). These variations were mostly
203 substitutions (96.6%) and were mostly located in targeted regions (70.4 %). The mean
204 distance between two variations was ~50 kb. Filtering these polymorphisms based on their
205 expected frequency variations across F0 and F2 conditions in each line (see methods for
206 filtering conditions) allowed identifying 302 (1.23%) differential polymorphisms associated
207 with insecticide resistance across all lines. Most of them were line-specific with only three of
208 them shared between the Feni and Bend lines. Combining allele frequency filtering with a
209 F_{ST}-based Bayesian approach allowed detecting multiple genomic regions carrying
210 differential polymorphisms and showing low Q values in both F0 and F2 samples in any line
211 (Fig. 4 and S3 Table). Most of them were located in proximity of genes potentially involved in
212 metabolic resistance and included genes carrying non-synonymous variations associated
213 with resistance (see below). Among these regions, 5 were located on chromosome 1,
214 including two GST and 1 P450 clusters. The P450 cluster (3 CYP304 genes at ~287 Mb)
215 showed a pronounced selection signature for the Delt and Bend lines while the GST cluster
216 located at ~300 Mb appeared more associated with the Bend line. Several regions were also
217 detected on chromosome 2. One ABC transporter cluster (4 genes at ~90 Mb), 1
218 sulfotransferase cluster (2 genes at 134.15 Mb) and 2 CCE clusters (6 genes at ~174 Mb
219 and 4 genes at ~214 Mb) showed strong selection signature in the Feni line. The large GST
220 cluster (15 GSTE genes at ~351.5 Mb) was associated with both Feni and Delt lines while
221 the large CYP6 cluster (16 genes at ~419.2 Mb) showed a strong selection signature in all
222 lines. Among regions identified in chromosome 3, the 2 large P450 clusters (21 CYP325
223 genes at ~111.6 Mb and 18 CYP9J genes at ~368.5 Mb) and the sulfotransferase cluster (6
224 genes at 396.8 Mb) were detected in all lines. Despite the 81 polymorphisms detected in the
225 voltage-gated sodium channel gene carrying kdr mutations (gene *AAEL023266* ~316 Mb),

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226 only a moderate selection signature was detected in this region mainly in the Delt and Bend
227 lines.
228
229 Among all differential polymorphisms associated with resistance identified, 50 were non-
230 synonymous (Fig. 5 and S3 Table). Most of them affected detoxification genes located in
231 genomic regions showing strong selection signatures. All of them were line-specific except
232 the I324V mutation identified in the alcohol dehydrogenase gene *AAEL026142* found
233 associated with resistance in both Bend and Feni lines. Seven were associated with
234 resistance in the Delt line. These affected the alcohol dehydrogenase *AAEL020054* and the
235 P450 *AAEL001960* in chromosome 2 together with 4 clustered P450s from the CYP325
236 family and 1 sulfotransferase in chromosome 3. Ten were associated with resistance in the
237 Bend line affecting 8 distinct genes: 5 on chromosome 1, affecting the alcohol
238 dehydrogenase *AAEL026142*, the P450s *CYP9AE1* and *CYP329B1*, and *GSTD6*; 2 on
239 chromosome 2, affecting 2 CYP6 belonging to a large cluster of P450s (*CYP6M9* and
240 *CYP6N13*); and 3 on chromosome 3, affecting the P450s *CYP4K3* (2 variations) and
241 *CYP6AG3*. Finally, more than 30 non-synonymous variations were associated with
242 resistance in the Feni line affecting 21 distinct genes. On chromosome 1, this included the
243 alcohol dehydrogenase *AAEL026142* and *GSTD1* for which a coding frameshift was
244 negatively associated with resistance. Multiple isolated genes were affected on chromosome
245 2, including 1 ABC transporter, a few P450s and 1 UDPGT. Two CCE clusters located within
246 regions showing strong selection signatures (at ~174 Mb and ~214 Mb) were also affected
247 with the first cluster being affected by a total of 17 point mutations. Three P450s (*CYP6M11*,
248 *CYP6Y3* and *CYP6N13*) located within a large CYP6 cluster (at ~419 Mb) were also affected
249 on chromosome 2. Finally, only the P450 *CYP325T1* and the CCE (*AAEL001517*) were
250 affected on chromosome 3.
251

252 ## Discussion

253 Natural populations experience a variety of selective pressures often leading to the
254 expression of complex adaptive phenotypes. In mosquitoes transmitting human diseases, an
255 over-reliance on chemical control has resulted in the rapid selection and spread of alleles
256 conferring resistance to various insecticides, often leading to multi-resistance phenotypes [6,
257 11, 29]. As opposed to target-site mutations which are specific to a given insecticide mode of
258 action, the complexity and redundancy of insect detoxification systems underlying metabolic
259 resistance make it less predictable and can lead to the selection of various and multiple
260 resistance alleles depending on the local context [6, 30]. Most insecticide resistance studies
261 using field mosquito populations focused on resistance mechanisms to a given insecticide or

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262 a given chemical family. However, these kinds of studies did not fully discriminate alleles
263 associated with resistance to different insecticides, which may lead to false positives. In this
264 context, the present study attempted at demonstrating that the identification of resistance
265 alleles can be improved by combining simple genetic crosses and targeted DNA pool
266 sequencing, while maintaining experimental work and sequencing costs at reasonable levels.
267

268 Using controlled crosses for segregating resistance alleles to different 269 insecticides

270 Bioassays confirmed the high resistance of the F0 Guy-R composite population from French
271 Guiana to deltamethrin and its moderate resistance to the two other insecticides. As
272 expected, the introgression of susceptible alleles by controlled crosses strongly reduced
273 deltamethrin resistance in F2 individuals. This suggests that resistance alleles approaching
274 fixation in the initial F0 population were less present in F2 individuals, thus facilitating their
275 dose-response segregation. This was confirmed by the strong decrease of kdr mutations
276 frequencies observed between F0 and F2 individuals in each line. Cross-resistance patterns
277 obtained from F2 individuals supported the partial segregation of resistant alleles between
278 each line. This segregation was also supported by the divergent kdr mutations frequency
279 patterns observed from F0 to F2 conditions between lines. Nevertheless, an incomplete
280 segregation of resistance alleles was expected as only two generations of recombination are
281 likely not enough to break out genetic associations between alleles conferring resistance to
282 distinct insecticides in all individuals. Such partial segregation may also indicate that
283 particular genes/alleles are contributing to resistance to multiple insecticides. This was
284 previously shown in *Anopheles* mosquitoes and *Drosophila melanogaster* where particular
285 detoxification enzymes have been shown to metabolize multiple insecticides from different
286 chemical families [31-34].

287

288 Detoxification enzymes CNV associated with resistance

289 Metabolic resistance is frequently associated with the over-expression of detoxification
290 enzymes having the ability to degrade and/or sequester insecticides [6, 17]. Although
291 changes in gene expression can be caused by cis- or trans-mediated transcriptional or post-
292 transcriptional regulation, CNV may also impact gene expression. Initially associated with
293 organophosphate resistance in *Culex pipiens* [35], recent genomic studies confirmed the
294 involvement of CNV in metabolic resistance to various insecticides in mosquitoes [26, 27,
295 36]. Such key role of CNV in metabolic resistance is not surprising as the locus mutation rate
296 is far higher for CNV than for mutation [37]. In addition CNV events are favored by the
297 presence of transposable elements which account for a large part of most mosquito

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298 genomes [~50% in Ae. aegypti genome, 38]. Furthermore, CNV have a direct impact on
299 gene expression level (*i.e.* gene dosage effect) without necessarily altering protein function
300 [39], suggesting that advantageous duplications have moderate costs and can be rapidly
301 selected in natural populations undertaking strong insecticide pressures. Finally, it has been
302 shown in yeast that a specific environmental change can stimulate the occurrence of CNV
303 affecting genes involved in this specific adaptation [40]. Considering that the proposed
304 transcriptional mechanism depends on promoter activity and that detoxification enzymes are
305 frequently inducible by insecticides, such mechanisms may have also favored the selection
306 of CNV-mediated metabolic resistance to insecticides in mosquitoes.

307 Our study identified 39 detoxification genes affected by CNV in association with resistance to
308 insecticides. Although the F0 Guy-R composite population from French Guiana exhibits a
309 high resistance to the pyrethroid deltamethrin, only few CNV were found associated with
310 resistance to this insecticide and most of them did not show a strong dose-response in F2
311 individuals. Such low CNV signal was likely caused by the presence of kdr mutations which
312 are known to be of significant importance in deltamethrin resistance in *Ae. aegypti* [11, 18,
313 22]. This was confirmed by the high kdr mutations frequencies found in individuals surviving
314 to high dose of deltamethrin. However, our data also supported the added value of an
315 increased gene copy number of detoxification enzymes in deltamethrin resistance, especially
316 in F0 survivors for which kdr mutations are nearly fixed. This trend was particularly apparent
317 for CNV affecting a cluster of 4 CYP6 genes located on chromosome 1 and for a large
318 cluster of CYP9J genes located on chromosome 3. Indeed the over-expression of P450s
319 belonging to these two gene clusters was previously associated with pyrethroid resistance in
320 *Ae. aegypti* [11, 18, 24] and in *Aedes albopictus* [41]. Some of these genes, such as
321 *CYP6BB2*, *CYP9J28* and *CYP9J32* have been functionally validated as able to metabolize
322 pyrethroid insecticides [42, 43] and CNV contributing to the over-expression of these P450s
323 were previously identified in deltamethrin-resistant populations [26, 27]. Finally, the role of
324 the over-expression of other detoxification enzymes such as GSTs in pyrethroid resistance
325 has been previously suggested [44-47]. These findings are in accordance with our
326 identification of CNV affecting the microsomal GST *AAEL006818* and *GSTI1* in association
327 with deltamethrin resistance.

328 As compared to deltamethrin, more genes were affected by CNV associated with bendiocarb
329 and fenitrothion resistance. Indeed, even though resistance levels to these two insecticides
330 were lower, the absence of resistance mutations affecting the target of these insecticides in
331 *Ae. aegypti* because of genetic constraints [28] may have strengthened the association of
332 CNV with resistance.

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333 Among genes affected by CNV associated with bendiocarb resistance, the CYP6
334 *AAEL009018* located on chromosome 1 showed a strong and specific dose-response
335 association with bendiocarb survival in both F0 and F2. The over-transcription of this gene
336 was previously identified in multi-resistant populations from the Caribbean [48] but also in
337 Malaysian populations showing cross-resistance between pyrethroids and carbamates [49].
338 The weak association of this gene with deltamethrin and fenitrothion resistance observed in
339 our study supports its role in carbamate resistance. Similarly, CNV affecting the CYP6
340 *AAEL017061* showed a strong and specific association with bendiocarb resistance. The role
341 of this gene in resistance is supported by the strong association of CNV affecting its *An.*
342 *gambiae* orthologue *CYP6AA1* with insecticide resistance [50] but also by the capacity of *An.*
343 *funestus CYP6AA1* to metabolize bendiocarb [51].

344 Several CNV affecting various detoxification enzymes were associated with resistance to
345 fenitrothion. The genes *CYP6N17*, *CYP6Z8* and *CYP6M5*, *GSTX2* and the UDPGT
346 *AAEL000687* were specifically associated with fenitrothion resistance. Noteworthy,
347 orthologous P450s, GSTs and UDPGTs were also found highly over-transcribed in a Greek
348 *Aedes albopictus* strain selected with the organophosphate temephos [52] supporting their
349 potential contribution to organophosphate resistance. The amplification of a CCE cluster
350 known to play a key role in temephos resistance [53] was not detected in our study, most
351 likely because this CCE amplification is not distributed in French Guiana as confirmed by
352 previous CNV data [26, 27].

353 Overall, the present study supports the contribution of CNV in the over-expression of
354 detoxification enzymes conferring insecticide resistance in mosquitoes. Although deciphering
355 their genomic architecture and their spatial dynamics in natural populations would require
356 further work, these CNV represent promising DNA markers for designing novel high-
357 throughput molecular assays to track metabolic resistance in the field.

358

359 Selection signatures and non-synonymous variations associated with 360 resistance

361 The combination of allele frequency filtering and F_{ST}-based selection signature detection
362 allowed identifying several genomic regions associated with insecticide resistance. These
363 regions are often located in close proximity to detoxification genes involved in insecticide
364 metabolism or previously found over-expressed in resistant populations [11, 18], supporting
365 the robustness of our approach. However, a few regions showing strong selection signatures
366 were identified near genes rarely associated with resistance in *Ae. aegypti*. This included
367 multiple P450s from the CYP325, CYP4 and CYP12 families but also GSTs, UDPGTs, ABC-

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368 transporters and sulfotransferases, which may all be potentially involved in insecticide
369 metabolism pathways. Most of these regions are located far from known resistance loci and
370 included detoxification genes carrying non-synonymous variations associated with
371 resistance. This suggests they may represent additional resistance loci possibly linked to the
372 selection of particular detoxification enzyme variants. Indeed, even though most studies on
373 metabolic resistance focused on the identification of over-expressed detoxification genes, the
374 selection of particular variants leading to an increased insecticide metabolism rate can also
375 contribute to the overall resistance phenotype as demonstrated in the malaria vector *An.*
376 *funestus* [31, 54]. Among genes carrying non-synonymous variations associated with
377 resistance, the deletion leading to a frame-shift coding in *GSTD1* is of particular interest.
378 Indeed, as expected if this GST contributes to insecticide metabolism, the functional allele
379 was specifically associated with resistance to fenithrothion in both F0 and F1 conditions. This
380 enzyme has been shown to catalyze DDT dechloration and to be expressed in detoxification
381 tissues in *An. gambiae* [55, 56], supporting its role in insecticide resistance. Also of interest
382 are the multiple non-synonymous variations associated with fenitrothion resistance affecting
383 a cluster of CCE genes located at 174 Mb in chromosome 2, of which one (*CCEae3A*,
384 *AAEL023844*) has been shown to sequester and metabolize the organophosphate temephos
385 in both *Ae. aegypti* and *Ae. albopictus* [53]. However, previous studies associated temephos
386 resistance to the over-expression of this CCE gene through increased gene copy number
387 [26, 57] while no CNV was detected for this gene in the present study. This suggests that the
388 selection of carboxylesterase variants may also contribute to organophosphate resistance.
389 This hypothesis is also supported by the previous identification of point mutations in
390 *CCEae3A* for which docking simulations predicted an impact on temephos binding [57].
391 Although none of these mutations were found associated with resistance in our study, some
392 mutations identified in these CCEs are located near the catalytic triad (*e.g.* I330M in
393 *CCEae3A* and D332G in *AEL005123*) or the active site (*e.g.* P293A in *AAEL019678*).
394 Altogether, the present study allowed identifying multiple detoxification genes located in
395 genomic regions under selection and carrying non-synonymous mutations associated with
396 resistance. Although further work is required to validate their association with the phenotype,
397 the present study paves the way for better understanding their **c**ontribution in insecticide
398 resistance.

399

400 **Conclusions**

401 Although insecticide resistance has often been described as a monogenic adaptation in
402 response to a strong selection pressure, it frequently results from the accumulation of
403 multiple physiological and metabolic changes often leading to complex phenotypes. Because

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404 of their nature, target-site mutations are usually well characterized in mosquitoes and can
405 typically be genotyped by simple PCR-based molecular assays [11, 18]. In contrast, genomic
406 changes associated with metabolic resistance are far more difficult to characterize for various
407 reasons: First, metabolic resistance alleles frequently co-occur with target-site mutations,
408 thus weakening their association with the overall resistance phenotype. Second, the
409 complexity and redundancy of insect detoxification pathways can lead to the selection of
410 multiple and diverse alleles through local adaptation. Third, increased insecticide metabolism
411 can be the consequence of multiple and additive genetic changes including non-synonymous
412 polymorphisms causing structural changes of detoxification enzymes but also up-regulation
413 and increased gene copy number, both enhancing their expression. Although massive
414 parallel sequencing appears as a powerful tool for untangling the complexity of the genetic
415 bases of metabolic resistance, it association with a well-thought experimental design can
416 help reducing both false negatives and false positives. Here, we demonstrated that
417 combining simple genetic crosses with pool targeted DNA-seq can enhance resistance allele
418 segregation and produce high coverage sequence data for identifying metabolic resistance
419 alleles while maintaining experimental work and costs at an acceptable level. Our results
420 also suggest that eliminating the effect of target-site mutations by controlled crosses or gene
421 editing should improve the power of genotype-phenotype association studies targeting
422 metabolic resistance alleles. Considering the global threat of insecticide resistance on vector
423 control and the decades that will be necessary for the full deployment of insecticide-free
424 strategies, untangling the genetic bases of insecticide resistance still represents a challenge
425 for controlling vector-borne diseases.

426

427 Materials and methods

428 Ethics statement

429 Blood feeding of adult mosquitoes was performed on mice. Mice were maintained in the
430 animal house of the federative structure Environmental and Systems Biology (BEeSy) of
431 Grenoble-Alpes University agreed by the French Ministry of animal welfare (agreement n° B
432 38 421 10 001) and used in accordance to European Union laws (directive 2010/63/UE). The
433 use of animals for this study was approved by the ethic committee ComEth Grenoble-C2EA-
434 12 mandated by the French Ministry of higher Education and Research (MENESR).

435 Mosquitoes

436 The multi-resistant composite *Ae. aegypti* population from French Guiana used in this study
437 consisted in a pool of 6 natural populations collected in 2016 in the following localities:

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438 Cayenne (North-East), Sinnamary (North-East), Saint-Laurent du Maroni (North), Apatou
439 (North-West), Maripasoula (West) and Saint-Georges (East). Each population was collected
440 as larvae from up to 5 breeding sites located within a 5 km range. These populations were
441 separately raised to the adult stage and blood fed to generate adults of the next generation.
442 The composite population was then created by pooling 1000 virgin adults of both sexes from
443 each population and breeding them together for 3 generations without insecticide selection in
444 order to homogenize genetic backgrounds. The resulting composite population (F0 Guy-R)
445 was used for controlled crosses.

446

447 **Controlled crosses**

448 Batches of virgin F0 Guy-R females were exposed to a dose killing 80% of individuals (LD_{80})
449 of 3 insecticides belonging to distinct chemical families: the pyrethroid deltamethrin, the
450 organophosphate fenitrothion and the carbamate bendiocarb (same exposure conditions as
451 for bioassays, see below). Females surviving to each insecticide were then crossed with the
452 fully susceptible strain Bora-Bora (Susc) in order to create three distinct lines (Fig. 6). For
453 each line, controlled crosses were repeated twice and consisted in mass-crossing 100 virgin
454 females having survived insecticide exposure (F0-$Delt_{LD80}$, F0-$Bend_{LD80}$, F0-$Feni_{LD80}$) with an
455 equal number of virgin males from the susceptible strain. For each line, F1 individuals were
456 allowed to reproduce freely and blood fed in order to generate F2 individuals. F2 individuals
457 from each line were then segregated based on their resistance phenotype by exposing 3
458 days-old females to two increasing doses of their respective insecticide (LD_{25} and LD_{75}). F0
459 and F2 individuals from each line, unexposed and surviving to insecticides, were used for
460 molecular analyses (Fig. 6).

461

462 **Bioassays**

463 All bioassays were performed on 3 days-old non blood fed females using WHO tests tubes
464 equipped with insecticide-impregnated filter papers following WHO guidelines [58]. First,
465 dose-response bioassays with deltamethrin, fenithrothion and bendiocarb were performed on
466 the F0 Guy-R composite population to assess its resistance phenotype and identify the LD_{80}
467 of each insecticide to be used for the initial F_0 segregation. These bioassays were conducted
468 with at least five doses of deltamethrin (0.05 % to 1%), fenitrothion (0.0125% to 0.4%) and
469 bendioacarb (0.2 to 2%) and an exposure time of 60 min. At least five batches of 20
470 mosquitoes were used for each insecticide dose. Mortality data were recorded after a 24h
471 recovery time and submitted to a probit analysis using the XL-Stat Excel module (Addinsoft,
472 France) for estimating LD values. Resistance ratios (RR_{50}) to each insecticide were

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473 computed from LD_{50} values obtained for each line as compared to the susceptible strain.
474 Resistance levels of F1 and F2 individuals from each line were obtained following the same
475 procedure.

476 Cross-resistance profiles of F2 individuals from each line to all insecticides were then
477 evaluated using single-dose bioassays. For each insecticide, the dose was calibrated in
478 order to obtain a mortality ranging from 20% to 40% in the corresponding F2 line. Doses
479 used were as follows: deltamethrin 0.05% for 60 min, bendiocarb 0.5% for 60 min,
480 fenitrothion 0.1% for 45 min. At least 4 batches of 20 three days-old non blood fed females
481 were used per line and insecticide. Mortality was recorded after a 24h recovery time and data
482 were expressed as mean % mortality ± SD. Resistance levels to each insecticide were
483 compared across lines using a generalized linear mixt model (binomial family) using R
484 version 3.5.2 (R Core development Team).

485

486 **Deep targeted DNA-sequencing**

487 **Sample preparation.** Deep targeted DNA pool sequencing was used to search for genomic
488 variations associated with insecticide resistance in each line. Genomic DNA was extracted
489 from 2 batches of 50 adult females from each condition (F0 GuyR, $F0_{LD80}$, $F2_{LD25}$ and $F2_{LD75}$,
490 see Fig. 6) using the PureGene kit (Qiagen) following manufacturer's instructions. DNA
491 extracts obtained from each batch were quality-checked on agarose gel, quantified using the
492 Qubit dsDNA Broad Range kit (Qiagen) and mixed in equal quantity in order to obtain a
493 single genomic DNA extract representative of 100 individuals for each condition.

494 **Capture libraries preparation and sequencing.** The capture of genomic regions of interest
495 was based on the SureSelect® target enrichment system (Agilent Technologies). Capture
496 library was designed based on Aaeg L3 genome assembly and Aaeg L3.3 annotation and
497 consisted in 54,538 overlapping RNA probes of 120 bp. Among them, 32,494 probes
498 targeted the exons and 1.5 kb upstream regions of 336 candidate genes with a mean
499 coverage of 4X. The remaining 22,044 probes targeted 7,348 unique 220 bp intergenic
500 regions equally spread over *Ae. aegypti* genome. Candidate genes included all known
501 detoxification enzymes (cytochrome P450s, glutathione S-transferases, carboxylesterases,
502 UDP-glycosyltransferases) together with other enzymes potentially involved in insecticide
503 biodegradation pathways and insecticide target proteins. Intergenic regions were defined in
504 order to cover > 95% of *Ae. aegypti* genome using the following criteria: target region size =
505 220 bp; optimal distance between 2 regions = 150 kb ± 10 kb; region distance to any
506 annotated gene > 5 kb; avoid repeated and redundant regions; avoid regions with GC
507 richness > 70% or single nucleotide richness > 50%; avoid regions with undefined

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508 nucleotides (N); do not consider supercontigs < 150 kb; avoid regions located within 75.5 kb
509 of supercontig boundaries. All genomic regions targeted by the study are detailed in S4
510 Table. Capture was performed with the SureSelect^{XT} Reagent kit (Agilent Technologies)
511 following the 'SureSelect^{XT} Target Enrichment System for Illumina Paired-end Sequencing
512 Library' protocol vB.4. Briefly, 3 μg of genomic DNA from each sample were fragmented
513 using a Bioruptor (Diagenode), purified, ligated to adaptors and amplified by PCR using
514 Herculase II DNA polymerase (Agilent Technologies). After QC of library size and quantity,
515 libraries were hybridized to biotinylated baits and purified using Dynal MyOne streptavidin
516 beads (Invitrogene). Captured DNA fragments were amplified, purified and multiplexed
517 before sequencing. Sequencing was performed on an Illumina NextSeq500 and generated
518 more than 300 million 75 bp paired reads with an average of 23.3 million reads per sample.
519 Reads were assigned to each sample (unplexing) and adaptors were removed. Reads
520 quality was checked for each sample using FastQC
521 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and reads were loaded into
522 Strand NGS v3.1.1 (Strand Life Science) for further analyses.

523 **Reads mapping and filtering.** In order to minimize false positives arising from mapping bias
524 in high-redundancy and low complexity regions, CNV were only identified from coding
525 regions. Reads were mapped against all Aaeg L5 exons using a padding of 35 bp and the
526 following parameters: minimum 90% identity, maximum 5% gap, mean insert size of 167 ±
527 30 bp, mismatch penalty = 4, gap opening penalty = 6, gap extension penalty = 1, clipping
528 penalty = 5, min align read length = 30, ignore reads with more than 5 matches, trim 3' end if
529 base quality < 25. Reads were then filtered on their quality metrics and mapping quality using
530 the following criteria: mean read quality ≥ 28, N allowed ≤ 2, alignment score ≥ 90, Mapping
531 quality ≥ 40, read length ≥ 35, remove non primary multiply mapped reads, remove inter-
532 chromosomal split reads. Finally, mate missing, translocated and duplicated reads were
533 removed. For polymorphisms analysis, reads were mapped against the whole Aaeg L5
534 genome in order to consider both genic and intergenic target regions and maximize genome
535 coverage for the detection of selection signatures. The same mapping and filtering
536 parameters as for CNV were applied.

537 **CNV detection.** The coverage of all exonic regions was computed and only regions showing
538 a mean coverage between 30 and 800 reads/bp in all samples and a length > 45 bp were
539 retained. For all remaining regions, the normalized coverage was used for computing a
540 relative copy number variation in each sample as compared to a common reference obtained
541 from all samples. Relative copy number values were averaged per gene and centering and
542 dimensionality reduction was applied to minimize stochastic variation. For each line, genes
543 were considered affected by CNV associated with insecticide resistance if their CNV profile

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544 satisfied the following conditions: CNV($F0_{LD80}$ - F0)>0.3 AND CNV($F0_{LD80}$ - F2)>0 AND
545 [CNV($F2_{LD25}$ - F2)>0.2 OR CNV($F2_{LD75}$ - F2)>0.2]. Basically, CNV associated with resistance
546 were expected to increase from F0 to F0 survivors, decrease from F0 survivors to F2 after
547 crossing with the susceptible strain, and increase in F2 survivors. No dose response
548 condition was applied for CNV in order to minimize the confounding effect of target site
549 mutations.

550 **Polymorphisms and selection signatures.** Variants were call against the whole Aaeg L5
551 genome using the following parameters: coverage > 30 in all conditions, confidence score cut
552 off = 100, ignore loci with homopolymer stretch > 4, ignore loci with average base quality ≤
553 15, ignore loci with strand bias ≥ 50 and coverage ≥ 50, ignore reads with mapping quality ≤
554 20, ignore variants with less than 4% supporting reads. Among all variants called only those
555 polymorphic among our conditions (*i.e.* showing ≥ 5 % variation in at least two conditions)
556 were retained and their genic effects were computed.

557 Associations between polymorphisms and resistance to each insecticide were assessed by
558 combining an allele frequency filtering approach with an F_{ST}-based approach.

559 The frequency filtering approach was based on the expected resistance allele frequency
560 variations across conditions taking into account their initial frequency. Frequency thresholds
561 used are described in detail in Table 1. Basically, the frequency of alleles positively
562 associated with resistance was expected to increase from unexposed F0 individuals to F0
563 survivors, decrease from F0 survivors to unexposed F2 individuals (following crossing with
564 the susceptible strain), and increase again in F2 survivors in association with the insecticide
565 dose. Different initial allele frequency thresholds were used for identifying alleles associated
566 with deltamethrin (≥30% in F0 Guy-R) and those associated with bendiocarb and fenitrothion
567 resistance (≥15% in F0 Guy-R) to account for the higher deltamethrin resistance level of the
568 initial composite population. The frequency of deleterious alleles (*i.e.* those negatively
569 associated with resistance) was expected to behave reciprocally.

570 The F_{ST}-based approach aimed at assessing departure from neutrality using the Bayesian
571 method implemented in BayeScan version 2.1 [59]. Because substitutions and deletions may
572 have different probability of occurrence, only substitutions were considered for this analysis.
573 For each insecticide line, 2 analyses were run separately. The first one contrasted allele
574 frequencies in F0 samples (unexposed and insecticide survivors: F0 Guy-R and $F0_{LD80}$) and
575 the second one in F2 samples (unexposed and survivors to each insecticide dose: F2,
576 $F2_{LD25}$, $F2_{LD75}$). The Markov chain Monte Carlo (MCMC) algorithm was run with prior odds of
577 10. The proposal distributions for parameters were adjusted by running 20 short pilot runs of
578 2,000 iterations. A burn-in period of 100,000 iterations was used and the posterior

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579 probabilities were estimated from the following 500,000 iterations (10,000 iterations samples
580 every 50). Genomic regions showing low Bayscan *Q*-values in both F0 and F2 comparisons
581 and including differential polymorphisms identified from the frequency filtering approach were
582 considered as under selection in association with insecticide resistance.

583

584 **Table 1.** Conditions used for identifying polymorphisms associated with resistance

Line[2]	Initial allele frequency (%)[1]	Minimum allele frequency variation[1]				
		F0 to $F0_{LD80}$	$F0_{LD80}$ to F2	F2 to $F2_{LD25}$	$F2_{L25}$ to $F2_{LD75}$	F2 to $F2_{LD75}$
Delt	30 to 85	+ 15 %	decrease	increase	increase	+ 15 %
	85 to 90	+ 10 %				
	90 to 95	+ 5 %				
	> 95	increase				
Bend Feni	15 to 85	+ 15 %				
	85 to 90	+ 10 %				
	90 to 95	+ 5 %				
	> 95	increase				

[1] Reciprocal conditions were used for deleterious alleles.

[2] different initial allele frequency threshold were chosen for Bend and Feni lines to account for the lower resistance of the initial F0 Guy-R population to these two insecticides.

585

586 **Kdr mutations genotyping**

587 Allelic frequencies for the three kdr mutations (V410L, V1016I and F1534C) initially present
588 in the F0 Guy-R composite population were obtained from F0 and F2 samples of each line
589 based on reads data. In order to validate allele frequencies obtained from targeted pool
590 DNA-seq, the two kdr mutations V1016I and F1534C were genotyped on individual
591 mosquitoes from the initial F0 Guy-R population (F0) together with F0 and F2 samples of the
592 Delt line. Total genomic DNA was extracted using cetyl trimethyl ammonium bromide
593 chloroform/isoamyl alcohol from 30 non blood fed females per condition as described in [60].
594 Individual genotypes for each kdr mutations were obtained by qPCR high resolution melt
595 curve analysis using 0.15 ng of genomic DNA per reaction as described in [20].

596

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597 ## Data availability statement

598 The sequence data from this study have been deposited to the European Nucleotide Archive
599 (ENA; http://www.ebi.ac.uk/ena) under the accession number PRJEB30945.

600

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604

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611

612 ## Author contributions

613 Conceived and designed the experiment: JPD. Performed experiments: FF, BL, MM, LG, TG,
614 FL, SR. Analyzed the data: JC, FF, BL, SS, SR, JPD. Collected biological material: FF, ID.
615 Wrote the manuscript: JC, JPD.

616

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813

814
Figure captions

815 **Fig 1. Insecticide resistance Levels.** Resistance levels of the different lines to the three
816 insecticides deltamethrin, bendiocarb and fenitrothion. Black: susceptible strain, Grey: F0
817 Guy-R composite population, Blue: Delt line, Purple: Bend line, Red: Feni line. A: Resistance
818 levels of each line to its respective insecticide at the F0 Guy-R, F1 and F2 generations.
819 Resistance levels are expressed as LD_{50} ± 95% CI. B: Cross-resistance profiles of each line
820 to all insecticides at the F2 generation. Cross-resistance levels are expressed as % mortality
821 ± SD to a single insecticide dose. For each insecticide, letters indicate statistical similarity or
822 dissimilarity between lines (GLM family = binomial, N≥4).

823

824 **Fig 2. Evolution of kdr mutations frequencies**. Allelic frequencies variations of the three
825 kdr mutations V410L, V1016I and F1534C initially present in the F0 Guy-R population in
826 each line. Allele frequencies were inferred from the number of sequencing reads supporting
827 each allele at each locus. Empty dots indicate conditions with read coverage < 30.

828

829 **Fig 3. Gene copy number variations associated with insecticide resistance.** For each
830 line, genes affected by CNV associated with resistance are indicated by stars. CNV data
831 obtained from F0 Guy-R are repeated for each line for better clarity. Black dots indicate
832 genes previously identified as affected by CNV associated with deltamethrin resistance in
833 Faucon et al. [26]. The genomic location of each gene on chromosomes and gene clusters
834 architecture is shown on the right (red: CNV associated with resistance, brown: CNV not
835 associated with resistance, grey: genes not included in the targeted regions). The Venn

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836 diagram indicates the number of genes affected by CNV associated with resistance in each
837 line.
838
839 **Fig 4. Selection signatures associated with insecticide resistance.** For each line, region
840 under selection were identified based on the presence of differential polymorphisms from
841 allele frequency filtering (red dots, see methods for filtering conditions) and the presence of
842 loci displaying low Q values in both F0 and F2 conditions. Q values were computed
843 separately in F0 (left arm) and F2 conditions (right arm) and Q values = 0 were fixed at 10^{-1000}
844 for better clarity. Horizontal grey lines indicate genomic regions showing strong selection
845 signatures associated with resistance. For each region the names of potential resistance
846 genes located within a 50 kb range are indicated. Grey dots indicate genomic regions
847 carrying genes affected by differential non-synonymous variations associated with resistance
848 as shown in Fig 5.
849
850 **Fig 5. Non-synonymous polymorphisms associated with insecticide resistance.** The
851 Venn diagram indicates the number of non-synonymous polymorphisms identified in each
852 line. The heat map shows allele frequencies in each condition. For each variation the allele
853 represented on the heat map is indicated in blue. Allele frequency data obtained from F0
854 Guy-R are repeated for each line for better clarity. For each line, stars indicate non-
855 synonymous polymorphisms associated with resistance based on their expected allele
856 frequency profile across all conditions. Grey dots indicate non synonymous polymorphisms
857 located in genomic regions showing strong selection signatures as shown in Fig 4. For each
858 variation the following annotations are shown: genomic location on chromosome, reference
859 nucleotide > variant nucleotide, amino acid position, amino acid change (fs= frame shift),
860 gene accession number, gene name.
861
862 **Fig 6. Experimental design overview.** Insecticide survival segregation steps are shown as
863 dashed arrows with the corresponding lethal dose (LD) indicated. Colors indicate insecticide
864 lines (blue: Delt-line, purple: Bend-line, red: Feni-line). The initial resistant composite
865 population (F0 Guy-R) and the susceptible strain (Susc) are shown in black and grey
866 respectively. Large dots indicate samples used for targeted DNA-seq.
867

868 Supplementary materials

869 **S1 Fig. Cross-validation of kdr mutation frequencies obtained by mass sequencing**
870 **and individual genotyping.** Blue dots show the allele frequency obtained by targeted DNA-
871 seq with the total read coverage indicated for each condition. Empty blue dots designate

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872 conditions for which the total read coverage was < 30. Triangles designate allele frequencies
873 obtained by qPCR genotyping with the number of genotyped individual indicated for each
874 condition.
875
876 **S1 Table. CNV data set.** This table shows the mean relative copy number of each gene
877 across all F0 and F2 conditions. Relative gene copy numbers were obtained by comparing
878 the normalized coverage of each target region to a common reference made from all
879 samples. Only regions showing a mean coverage between 30 and 850 in all samples were
880 considered. Relative copy numbers were then averaged per gene and centered-reduced in
881 order to minimize stochastic variations. Genes affected by CNV associated with resistance to
882 each insecticide are indicated.
883
884 **S2 Table. Polymorphism data set overview.** This table describes the overall
885 polymorphisms data set. Polymorphisms counts and percentages are given for the following
886 categories: passing QC filters and polymorphic across samples, differential in each line, non-
887 synonymous differential in each line.
888
889 **S3 Table. Polymorphism data set.** This table describes all variations passing QC filters and
890 polymorphic across samples. For each variation, the following attributes are listed: location
891 (chromosome, start, end), reference and variant allele, polymorphisms type, target region
892 type, variant allele frequency in each condition, differential based on allele frequency filtering
893 (Yes or No for each line), Bayescan -Log_{10} Q value (for F0 and F2 condition in each line),
894 genic effect based on AaegL5.1 annotation (effect, affected gene accession and description,
895 AA change, cDNA position/effect, protein position/effect).
896
897 **S4 Table. Genomic regions targeted by DNA-seq.** This table describes all genomic
898 regions targeted by DNA-seq. For each target region, the following information are shown:
899 AaegL5 location (chromosome, start, end, length), AAegL3 location (supercontig: start-end,
900 length), marker type (genomic “marker”, gene “promoter” or “gene”), short description,
901 protein family.
902

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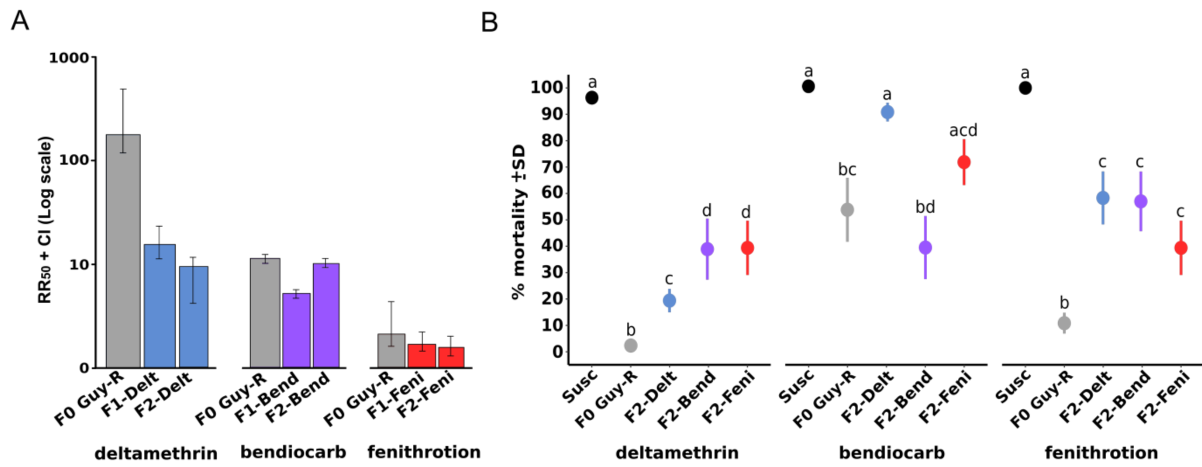
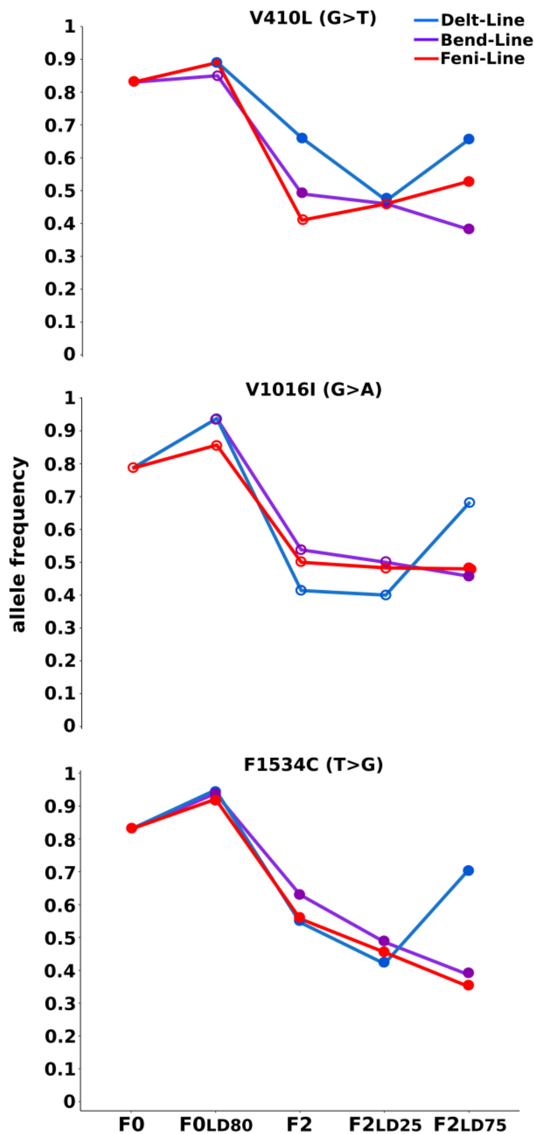
903 **Figure 1.**



904

905 **Figure 2.**



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925 **Figure 3.**

926

927

928 **Figure 4.**

929

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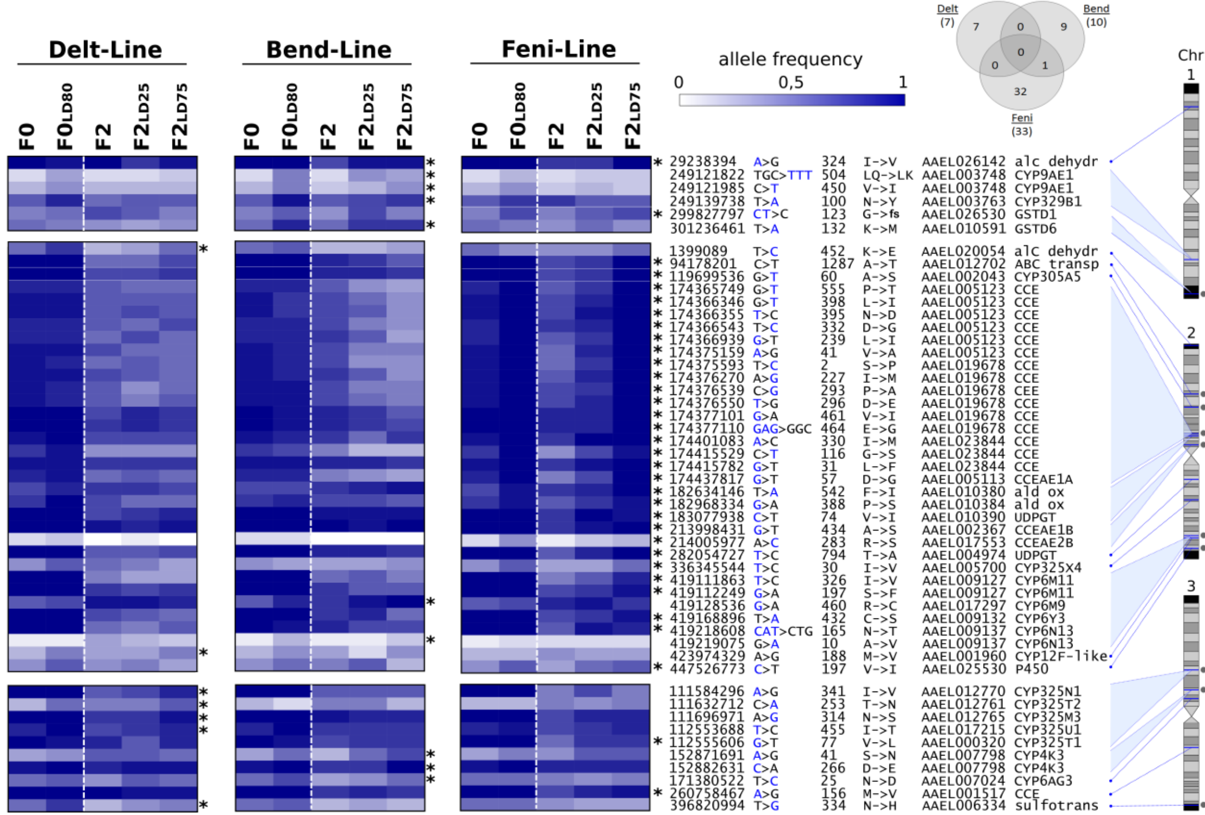
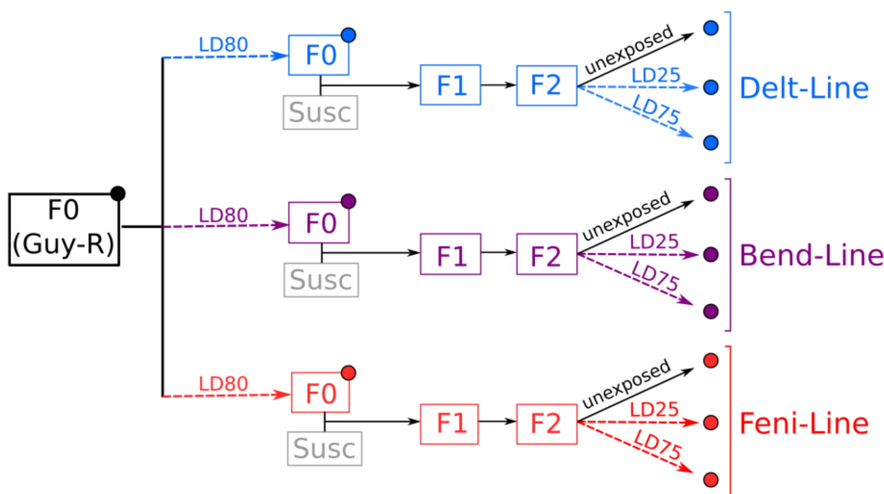
930 **Figure 5.**



931

932

933 **Figure 6.**



934

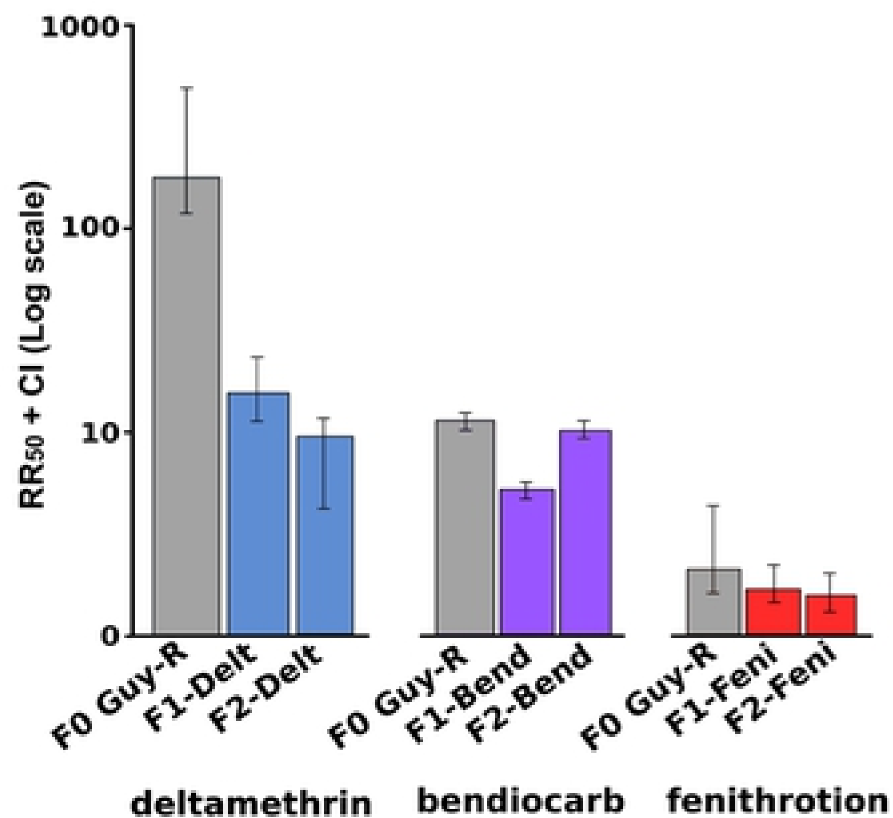
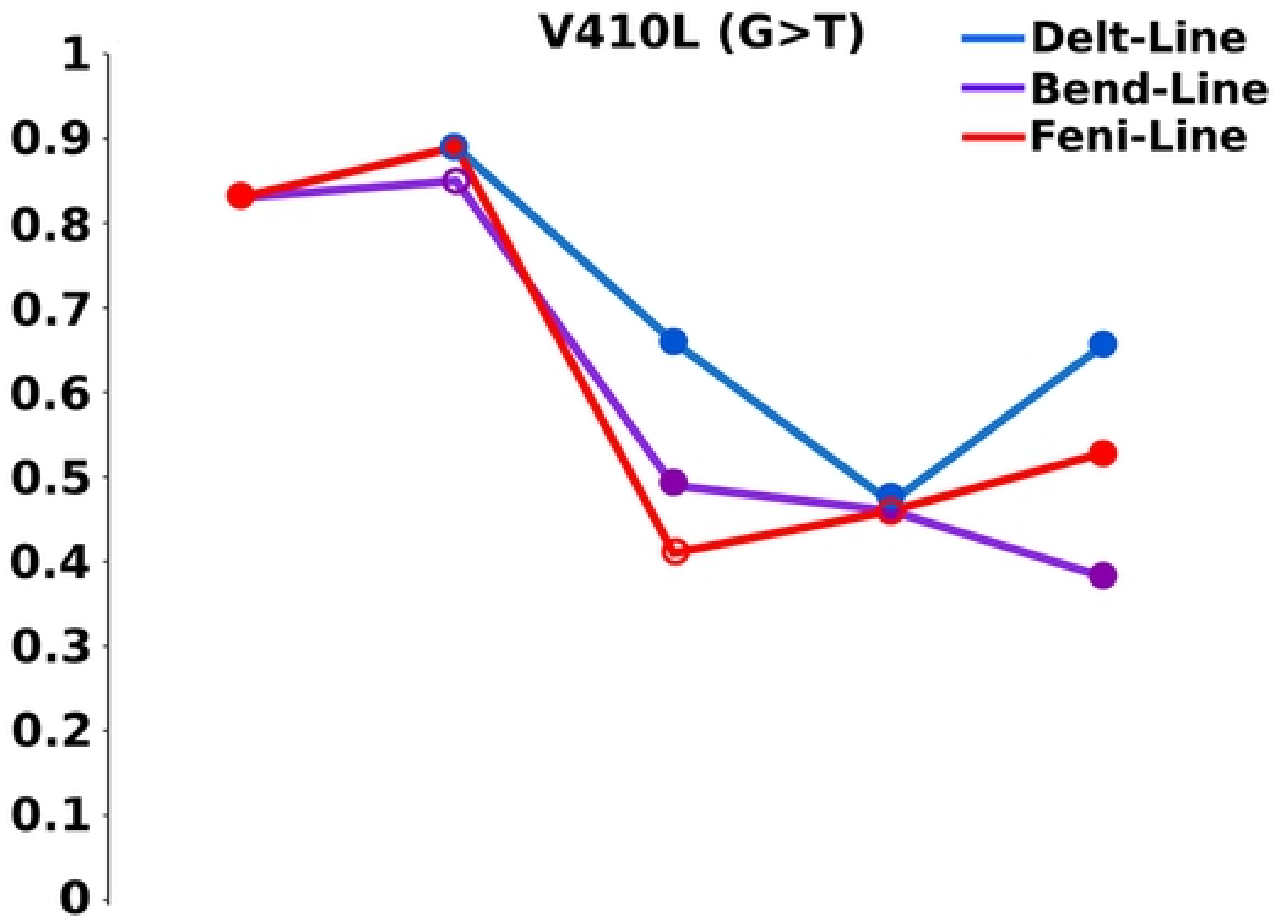


Figure 1 (high resolution)



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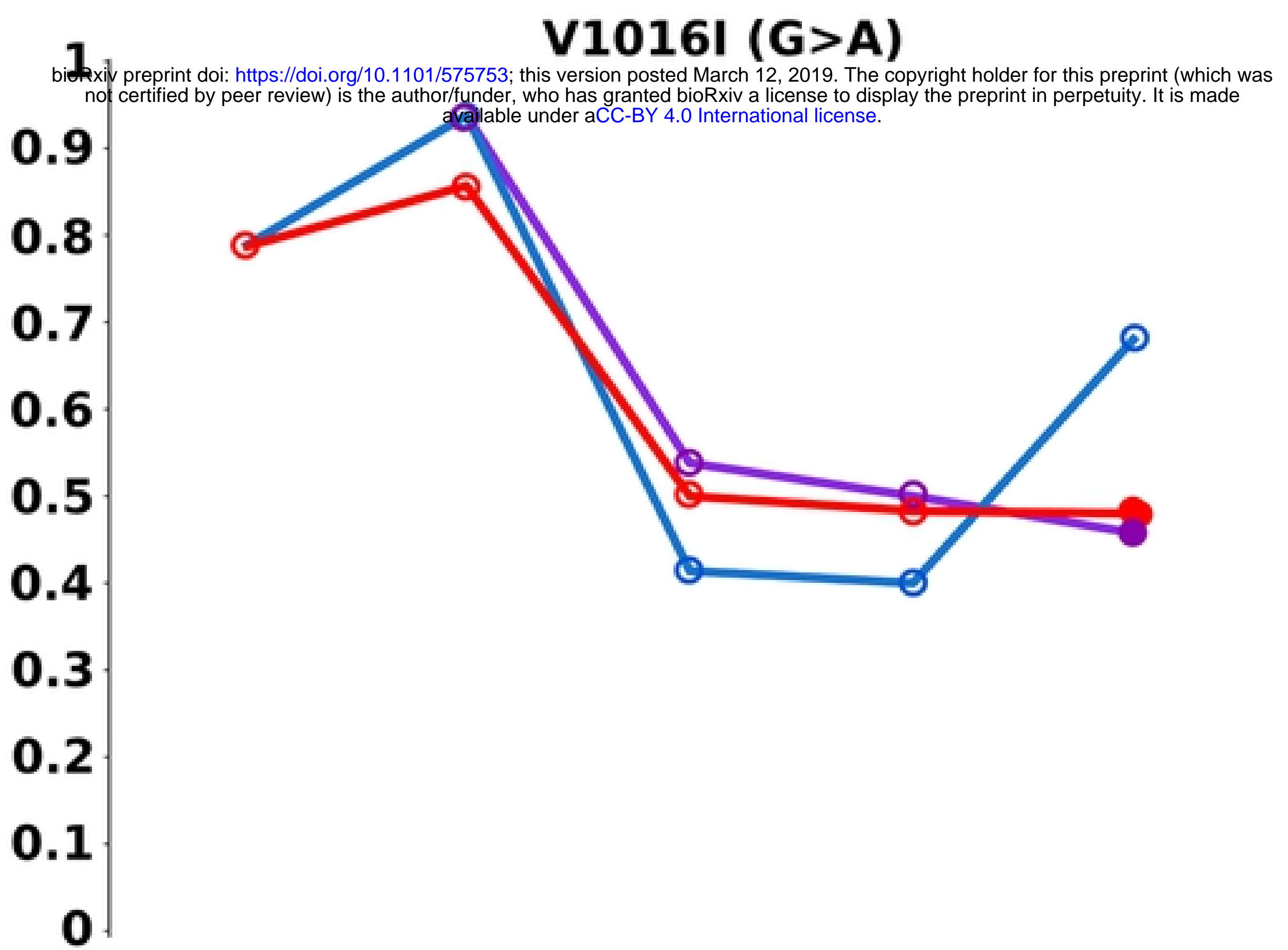

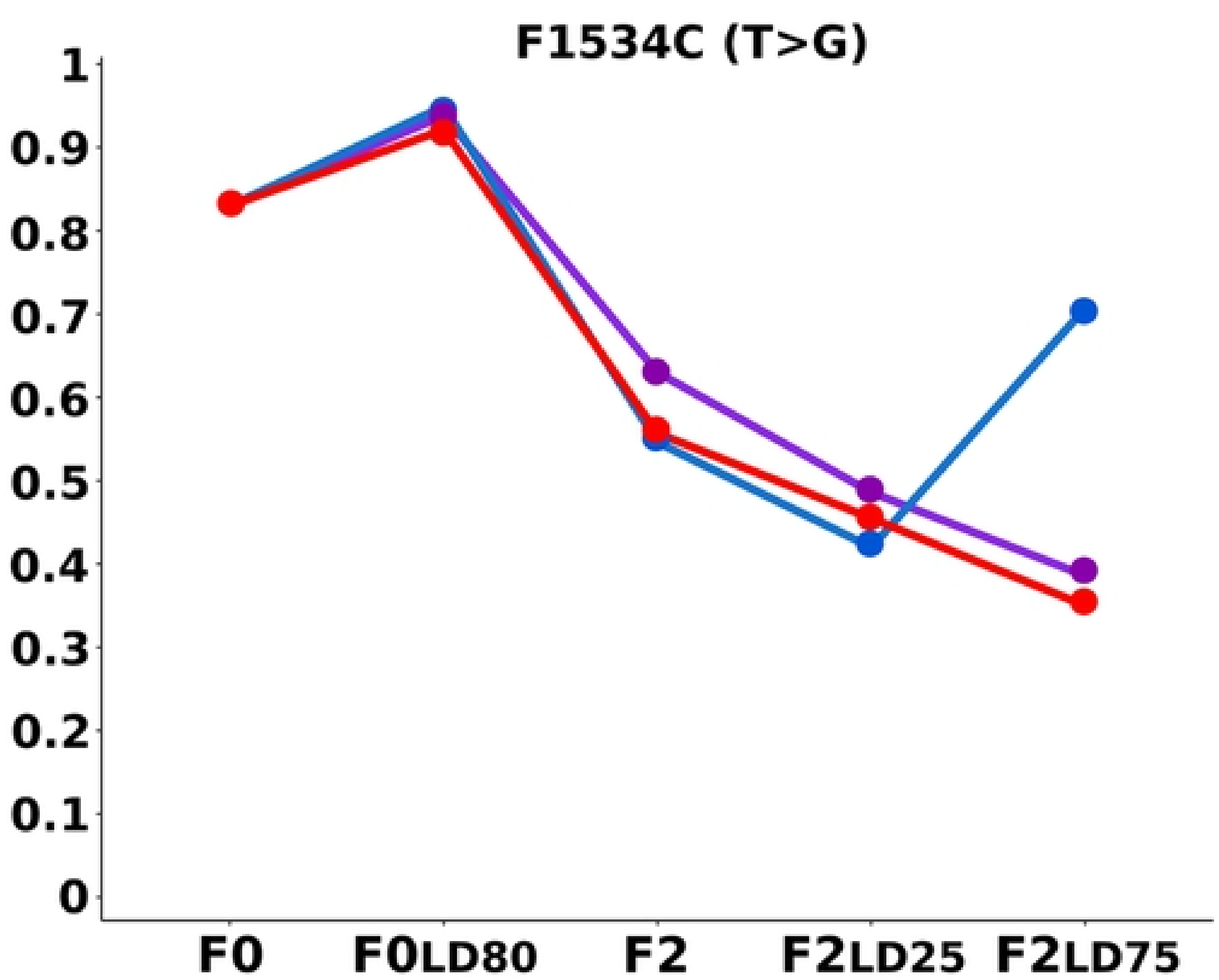

Figure 2 (high resolution)

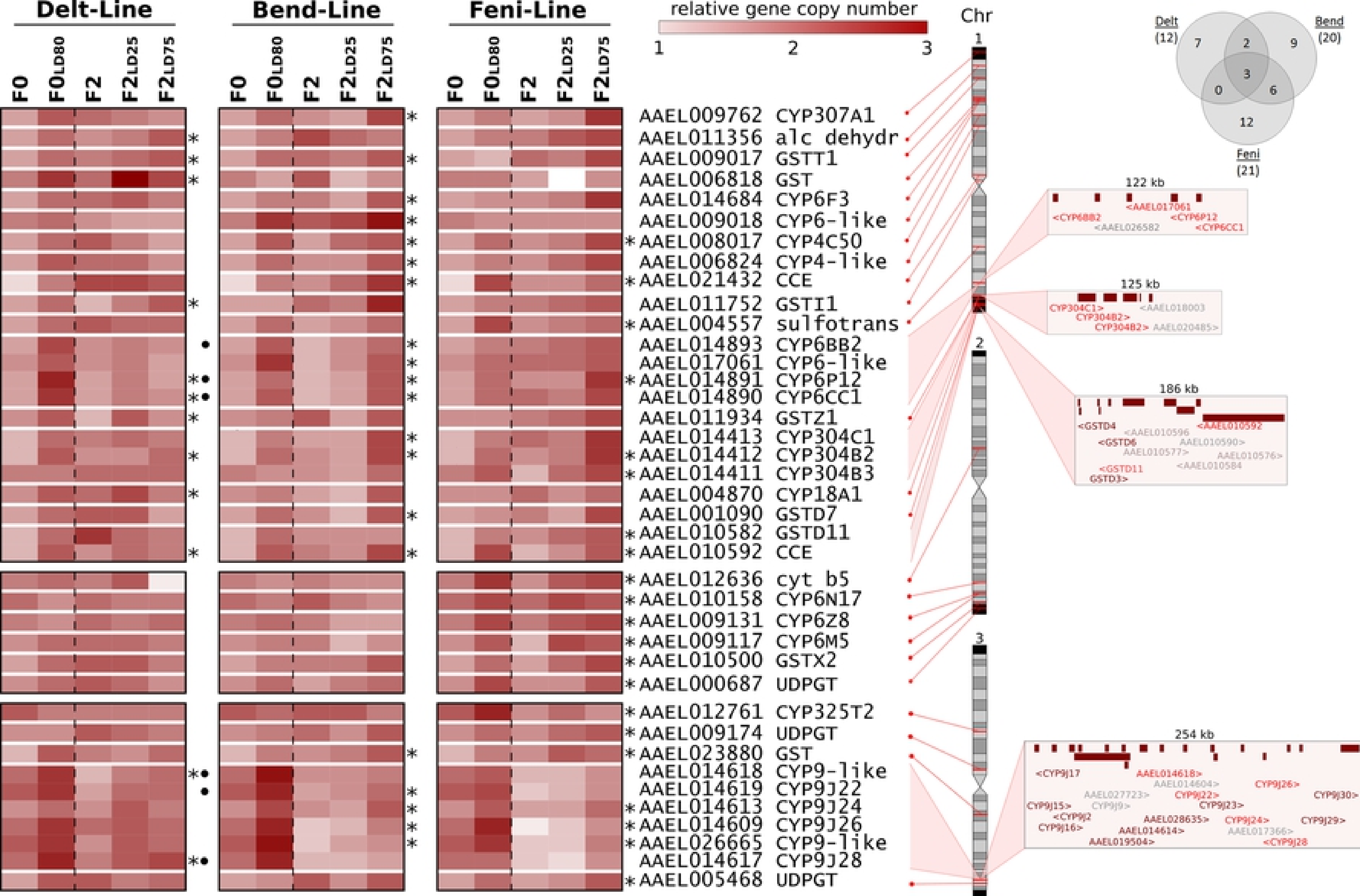

Figure 3 (high resolution)

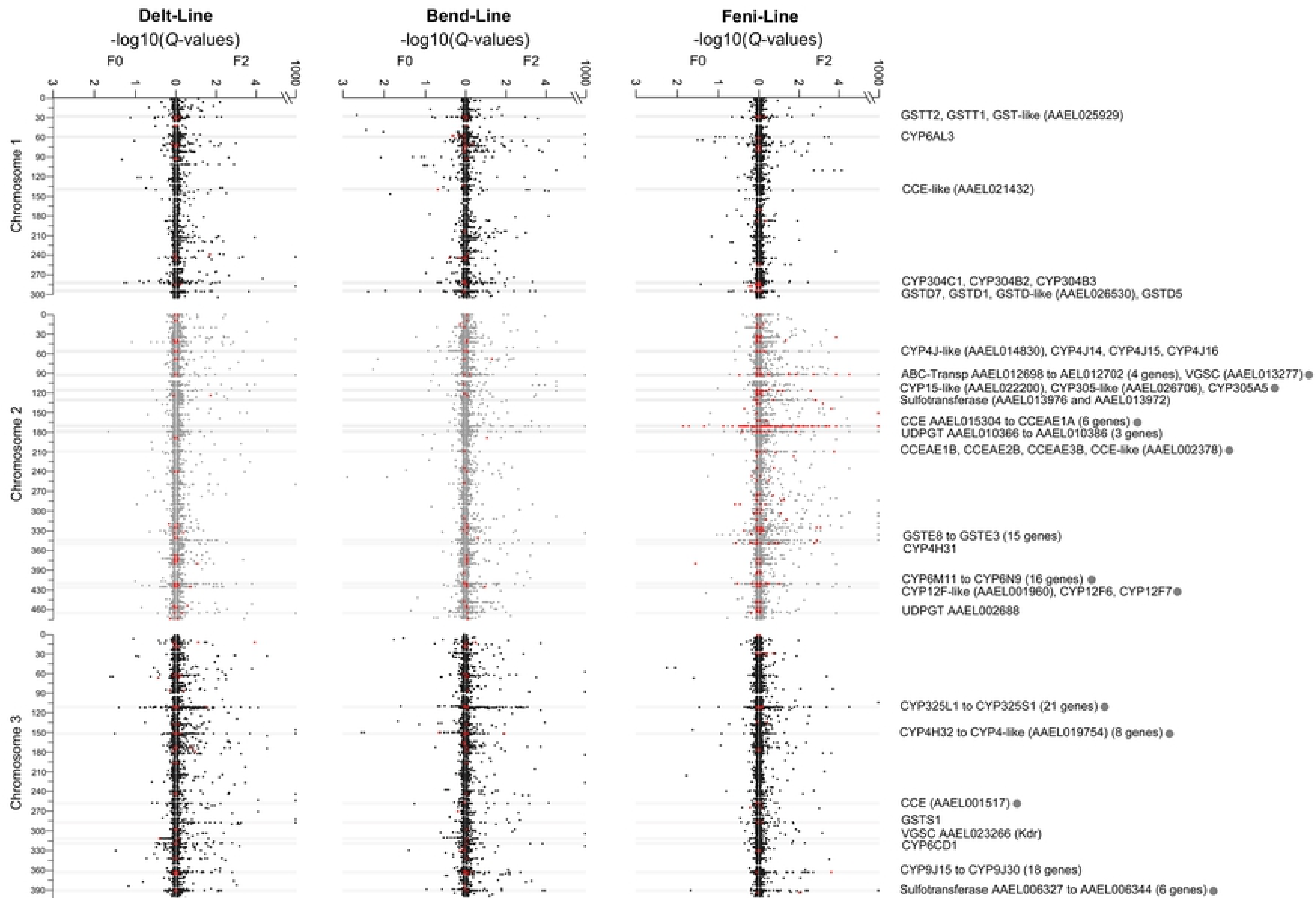

Figure 4 (high resolution)

Delt-Line | Bend-Line | Feni-Line

F0 | F0LD80 | F2 | F2LD25 | F2LD75

allele frequency 0 0,5 1

Delt (7) 7 | Bend (10) 9 | Feni (33) 32 | 0 | 0 | 0 | 1

Chr 1 2 3

Position	Change	Codon	AA change	Gene ID	Annotation
29238394	A>G	324	I->V	AAEL026142	alc dehydr
249121822	TGC>TTT	504	LQ->LK	AAEL003748	CYP9AE1
249121985	C>T	450	V->I	AAEL003748	CYP9AE1
249139738	T>A	100	N->Y	AAEL003763	CYP329B1
299827797	CT>C	123	G->fs	AAEL026530	GSTD1
301236461	T>A	132	K->M	AAEL010591	GSTD6
1399089	T>C	452	K->E	AAEL020054	alc dehydr
94178201	C>T	1287	A->T	AAEL012702	ABC transp
119699536	G>T	60	A->S	AAEL002043	CYP305A5
174365749	G>T	555	P->T	AAEL005123	CCE
174366346	G>T	398	L->I	AAEL005123	CCE
174366355	T>C	395	N->D	AAEL005123	CCE
174366543	T>C	332	D->G	AAEL005123	CCE
174366939	G>T	239	L->I	AAEL005123	CCE
174375159	A>G	41	V->A	AAEL005123	CCE
174375593	T>C	2	S->P	AAEL019678	CCE
174376270	A>G	227	I->M	AAEL019678	CCE
174376539	C>G	293	P->A	AAEL019678	CEE
174376550	T>G	296	D->E	AAEL019678	CCE
174377101	G>A	461	V->I	AAEL019678	CCE
174377110	GAG>GGC	464	E->G	AAEL019678	CCE
174401083	A>C	330	I->M	AAEL023844	CCE
174415529	C>T	116	G->S	AAEL023844	CCE
174415782	G>T	31	L->F	AAEL023844	CCE
174437817	G>T	57	D->G	AAEL005113	CCEAE1A
182634146	T>A	542	F->I	AAEL010380	ald ox
182968334	G>A	388	P->S	AAEL010384	ald ox
183077938	C>T	74	V->I	AAEL010390	UDPGT
213998431	G>T	434	A->S	AAEL002367	CCEAE1B
214005977	A>C	283	R->S	AAEL017553	CCEAE2B
282054727	T>C	794	T->A	AAEL004974	UDPGT
336345544	T>C	30	I->V	AAEL005700	CYP325X4
419111863	T>C	326	I->V	AAEL009127	CYP6M11
419112249	G>A	197	S->F	AAEL009127	CYP6M11
419128536	G>A	460	R->C	AAEL017297	CYP6M9
419168896	T>A	432	C->S	AAEL009132	CYP6Y3
419218608	CAT>CTG	165	N->T	AAEL009137	CYP6N13
419219075	G>A	10	A->V	AAEL009137	CYP6N13
423974329	A>G	188	M->V	AAEL001960	CYP12F-like
447526773	C>T	197	V->I	AAEL025530	P450
111584296	A>G	341	I->V	AAEL012770	CYP325N1
111632712	C>A	253	T->N	AAEL012761	CYP325T2
111696971	A>G	314	N->S	AAEL012765	CYP325M3
112553688	T>C	455	I->T	AAEL017215	CYP325U1
112555606	G>T	77	V->L	AAEL000320	CYP325T1
152871691	A>G	41	S->N	AAEL007798	CYP4K3
152882631	C>A	266	D->E	AAEL007798	CYP4K3
171380522	T>C	25	N->D	AAEL007024	CYP6AG3
260758467	A>G	156	M->V	AAEL001517	CCE
396820994	T>G	334	N->H	AAEL006334	sulfotrans

Figure 5 (high resolution)

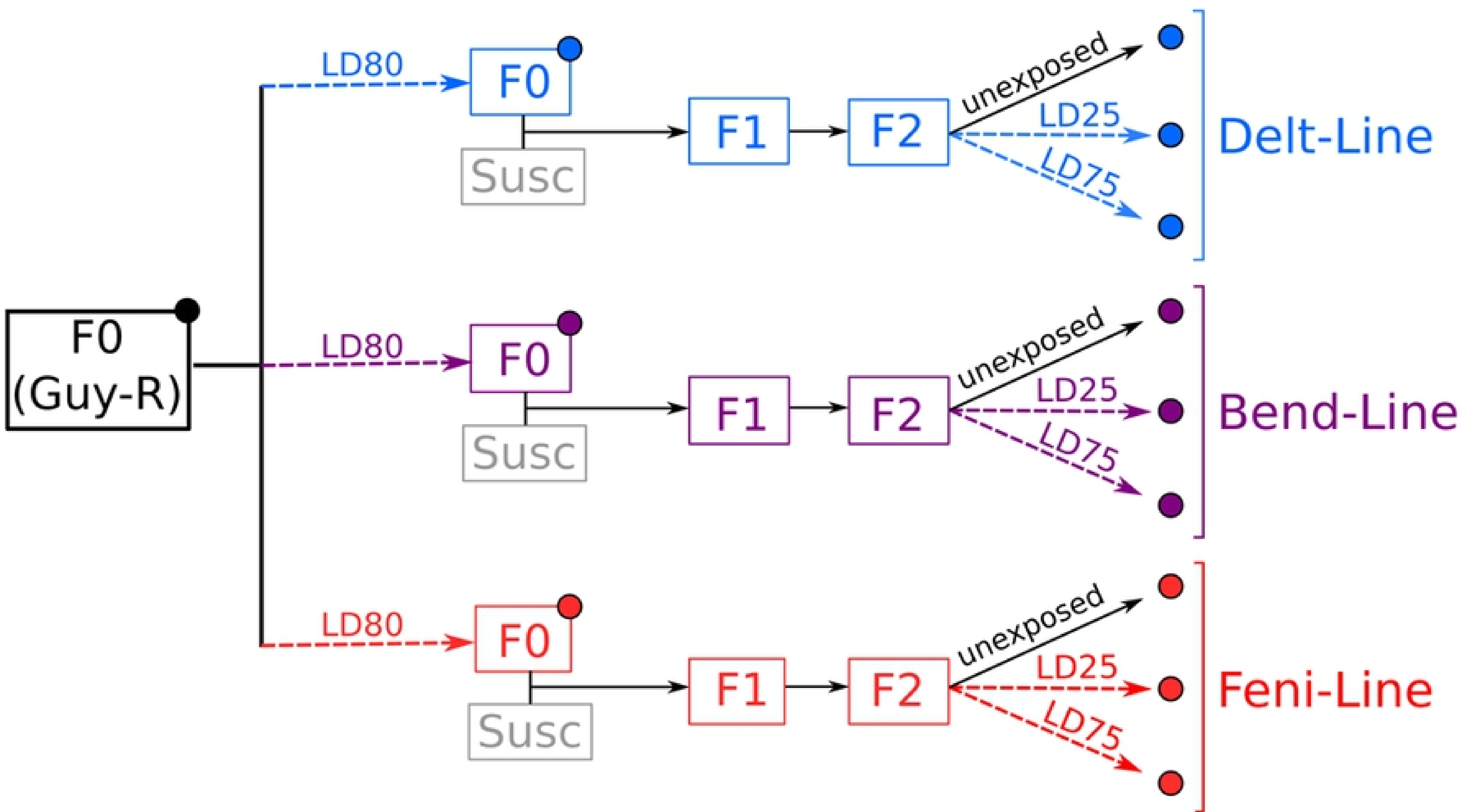

Figure 6 (high resolution)