Expansion of frozen hybrids in the guppy ectoparasite, *Gyrodactylus turnbulli*

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Abstract

Hybridization is one of the major factors contributing to the emergence of highly successful parasites. Hybrid vigor can play an important role in this process, but subsequent rounds of recombination in the hybrid population may dilute its effects. Increased fitness of hybrids can, however, be frozen by asexual reproduction. Here, we identify invasion of a "frozen hybrid" genotype in natural populations of *Gyrodactylus turnbulli*, a facultatively sexual ectoparasitic flatworm that causes significant damage to its fish host. We re-sequenced genomes of these parasites infecting guppies from six Trinidad and Tobago populations, and found surprisingly high discrepancy in genome-wide nucleotide diversity between islands. The elevated heterozygosity on Tobago is maintained by predominantly clonal reproduction of hybrids formed from two diverged genomes. Hybridization has been followed by spread of the hybrids across the island, implying a selective advantage compared to native genotypes. Our results thus highlight that a single outcrossing event may be independently sufficient to cause pathogen expansion.

Keywords: hybridization, clonal reproduction, heterosis, invasion, *Gyrodactylus*

Introduction

Hybridization refers to a successful mating between individuals from two populations, which are distinguishable on the basis of one or more heritable characters and have divergent genomes (Arnold, 2004). It can be a potent evolutionary force, creating opportunities for adaptive evolution by increasing genetic variation on which natural selection can act (Arnold, 1997, 2004; Oziolor et al., 2019; Rieseberg et al., 2003; Taylor & Larson, 2019). The ecological and evolutionary consequences are potentially large and may include significant effects on the evolutionary trajectories of pathogenic organisms ('parasites' henceforth) (King, Stelkens, Webster, Smith, & Brockhurst, 2015).

39 This role is likely to increase in importance because human activities increase opportunities for
40 hybridization by inducing environmental changes and incidental translocations (Brooks & Hoberg,
41 2007; Cable et al., 2017; Patz, Graczyk, Geller, & Vittor, 2000). Understanding the role of
42 hybridization in parasite evolution is therefore essential if we are to predict hot spots of disease risk
43 and emergence (Laine, Barrès, Numminen, & Siren, 2019).

44 Parasite strains of hybrid origin can gain an advantage because recombinants – individuals with
45 shuffled genomes - produced after hybridization may show enhanced phenotypic characteristics
46 compared to the parental generation, such as higher infectivity (Grigg, Bonnefoy, Hehl, Suzuki, &
47 Boothroyd, 2001), expanded host range (Detwiler & Criscione, 2010), increased transmission
48 potential (Volf et al., 2007) or increased tolerance to harsh environmental conditions (Laine et al.,
49 2019). Hybridization, however, is often associated with asexual reproduction, which may in the long
50 term preclude the initial benefits resulting from recombination between divergent genomes, since
51 asexual lineages tend to be less adaptable to environmental change compared to sexually
52 reproducing lineages (Neaves & Baumann, 2011). Asexual reproduction is thought to be enforced by
53 Bateson-Dobzhansky-Muller incompatibilities accumulated in parental lineages (Janko et al., 2018;
54 Neaves & Baumann, 2011). Asexual lineages frequently originate from hybridization events, for
55 example in cases when the genomes of parental species are divergent enough to disrupt meiosis in
56 hybrids, yet not diverged enough to seriously affect hybrid viability (Janko et al., 2018; Lamelza et al.,
57 2019; Moritz et al., 1989). Alternatively, the hybrid origin of asexual animals can arise from
58 hampered pairings of homologous chromosomes due to decreasing sequence homology (De Storme
59 & Mason, 2014) or from asynchronous expression of genes brought together by hybridization
60 (Carman, 1997).

61 Conversely, asexual reproduction may be selectively beneficial if clonal reproduction "freezes"
62 heterozygosity that confers short-term evolutionary advantage (Neiman, Sharbel, & Schwander,
63 2014). Hybrids often show greater vigor, productivity, disease resistance, or overall increased

64 Darwinian fitness (Kingsbury, 2009). Two alternative hypotheses may explain the existence of hybrid
65 vigor (heterosis) (Lippman & Zamir, 2007). The overdominance hypothesis, attributes heterosis to
66 superior fitness of heterozygous genotypes over homozygous wild types. The dominance hypothesis
67 assumes that recessive deleterious mutations are to some extent masked in a heterozygous stage.
68 Recent theoretical work indicates that dominance-induced heterosis might play an important role in
69 evolution, especially if deleterious alleles are not effectively removed from populations as it is
70 expected when populations are small (Gilbert, Pouyet, Excoffier, & Peischl, 2020; Kim, Huber, &
71 Lohmueller, 2018; Zhao & Charlesworth, 2016). This mechanism may be particularly relevant to
72 parasite evolution. Many parasite populations are small or experience frequent bottlenecks,
73 therefore are prone to the accumulation of deleterious mutations (M. Lynch, Butcher, Bürger, &
74 Gabriel, 1993), but also to elevated homozygosity due to inbreeding. In such a case, one could expect
75 higher fitness of hybrid parasites. Successful parasite strains originating from a hybridization event
76 have been documented in several species, including *Batrachochytrium dendrobatidis* (Farrer et al.,
77 2011; Greenspan et al., 2018), *Toxoplasma gondii* and *Sarcocystis neurona* (Grigg et al., 2001;
78 Wendte et al., 2010). However, because in all these cases some degree of recombination occurred,
79 the relative role of heterozygosity versus recombination between highly divergent genomes remains
80 unresolved.

81 Here, we report frozen genome-wide heterozygosity in wild *Gyrodactylus turnbulli*, a common guppy
82 parasite and well-known model species in epidemiological and eco-evolutionary research (e.g. Houde
83 & Torio, 1992; Jacquin et al., 2016; Phillips et al., 2018; Reynolds, Arapi, & Cable, 2018; van
84 Oosterhout, Harris, & Cable, 2003). Previous laboratory work demonstrated that *G. turnbulli* from
85 mixed strain infections often hybridize and have higher fitness (more parasites per host, longer
86 infection, but not increased host mortality), suggesting that in fact hybrid individuals might have
87 increased fitness, possibly due to heterosis or recombination (Schelkle, Faria, Johnson, van
88 Oosterhout, & Cable, 2012). However, the effect of hybridization on population structure and
89 dynamics of infections in wild populations has not been investigated to date (Xavier et al., 2015). We

analyze thirty whole genomes of *G. turnbulli* originated from six natural populations from Trinidad and Tobago. On Tobago we identified a widespread, recently formed "frozen hybrid" genotype, suggesting the potential importance of heterosis in clonal invasions of *G. turnbulli* and highlight that a single hybridization event can lead to the emergence of highly successful parasites, despite suppressing recombination.

Materials and Methods

Field collections

During two expeditions (2017 and 2018), guppies (*Poecilia reticulata*) were sampled from six locations on Trinidad and Tobago (Trinidad: Aripo River [ARI], Caura River [CAU], Lopinot River [LOP]; Tobago: Dog River [DR], Health Center [HC], Spring Site [SPS]). Fish were transported to our field station in Charlotteville, northeast Tobago, where each population was kept in a separate aquarium. Over four weeks, guppies were subsequently screened for ectoparasites under a dissecting microscope. If gyrodactylids were observed, a single worm was allowed to move to a naïve, anaesthetized fish, obtained from *Gyrodactylus*-free mesocosms established by Phillips et al. (2018). The transfer was closely monitored under the microscope to ensure movement of just a single worm. Fish experimentally infected in this way were kept in separate tanks in order to establish strains from which we could obtain enough DNA required to prepare libraries for sequencing. Fish were screened every 2-3 days to verify the presence of gyrodactylids, and hosts were euthanized with an overdose of MS-222 8-13 days post-infection. The number of worms was counted, samples were preserved in 97% molecular grade ethanol and transported to Poland. Life expectancy for *G. turnbulli* was estimated to 4.2 days (Scott, 1982). The first offspring is born approx. 1 day after the birth of the parent, and subsequent offspring are born at 2-2.5 days intervals (Scott, 1982). We thus expect that most individuals develop from the first or second births. The first-born always develops clonally, while the second-born daughter originates from parthenogenesis (Bakke, Cable, & Harris, 2007;

115 Cable & Harris, 2002). Even though it is unclear which mechanism restores the diploid component
116 during parthenogenesis (Cable & Harris, 2002), it has been suggested that heterozygosity is
117 maintained during that process, as reported for *G. salaris.* Third- and subsequent born daughters
118 may result from outcrossing, selfing, or parthenogenesis. Given our experimental design we expect
119 that the majority of gyrodactylid individuals on each experimentally infected fish developed from
120 first or second births and should have preserved founder's genomes. Samples with at least 10 worms
121 were used for DNA extraction and for species identification as described below. Parasites collected in
122 2017 and identified as *G. bullatarudis* were reported in a separate study (Konczal et al., 2020), while
123 this work focused solely on *G. turnbulli*.

124

125 *DNA extraction, libraries preparation and sequencing*

126 DNA was extracted from pools of individuals ('strain' henceforth) derived from a single worm
127 collected from a single experimentally infected fish using MagJET Genomic DNA kits (Thermo
128 Scientific). DNA concentration was measured using Qubit High Sensitivity reagents. *Gyrodactylus*
129 were identified to species using Sanger sequencing of a 262 bp fragment of the mitochondrial protein
130 coding COII gene (Xavier et al., 2015). Sequences were aligned together with records downloaded
131 from the NCBI Genbank, and a Neighbor Joining tree was reconstructed with MEGA X (Kumar,
132 Stecher, Li, Knyaz, & Tamura, 2018) to determine species. Thirty strains were selected for library
133 preparation (Nextera DNA Flex Library Prep Kit, Illumina) based on DNA quantity and population of
134 origin. Subsets of strains were sequenced in 2018 with HiSeq2500 (2 x 100 bp). All other strains were
135 sequenced in 2019 on the NovaSeq6000 platform in 2 x 150 bp mode (see Supplementary Table 1 for
136 more details).

137

138 *SNP calling and population genomic analyses*

139 The quality of raw reads was assessed with Fastqc (Andrews & Babraham Bioinformatics, 2010).
140 Reads were then filtered with trimmomatic (version 0.36; Bolger, Lohse, & Usadel, 2014) and
141 mapped using bwa mem (version 0.7.10-r789) to the reference nuclear genome (Hahn et al., in
142 prep.), which was assembled from individuals from the *Gt3* laboratory strain kept at Cardiff
143 University. Mapping results was visualized with Qualimap (García-Alcalde et al., 2012) and duplicated
144 reads were marked with picardtools (version 2.18.5-6). SNPs were then called with samtools mpileup
145 (version 1.6.0, options -R -C50 -t DP, ADF, ADR). Based on inspection of the distributions of diagnostic
146 statistics, we removed SNPs with DP > 1200 or DP < 400 (for all samples together), with QUAL < 30,
147 with MQ < 30 or in 5 bp distance from identified indels.

148 We then used SNPs to calculate PCA with plink (version 1.90, Purcell et al., 2007) using only SNPs
149 with minor allele frequency greater than 0.05. The same dataset with additional sample representing
150 reference genotype (all genotypes encoded as reference homozygous) was used to calculate distance
151 matrix (in one minus identity-by-state units) with plink and to construct a Neighbor Joining tree using
152 the ape package in R. In addition, we ran PCA analyses with SNPs, that were previously pruned for
153 Linkage Disequilibrium. We first pruned SNPs with –indep-pairwise 100 10 0.1 option in plink, and
154 then used the same program to calculate PCA (D. H. Alexander, Novembre, & Lange, 2009).

155 Vcftools (version v0.1.12b; Danecek et al., 2011) was used to calculate nucleotide diversity (π) and
156 genetic divergence between populations (F_{ST}) in 25 kb non-overlapping windows, as well as
157 inbreeding coefficient (F_{IS}, option –het) per strain. To calculate F_{IS} per strain we considered only SNPs
158 polymorphic within population of origin. The fraction of heterozygous sites per genomic window was
159 calculated using a custom script and our estimates of genome wide heterozygosity per strain were
160 inferred as the average of these fractions across all genomic windows. For each strain we also
161 calculated divergence form the reference genome in 25 kb non-overlapping windows, by counting
162 the number of non-reference variants (alternative homozygous sites were counted as one and
163 reference/alternative allele heterozygous sites were counted as half) and dividing it by a window

164 length. Windows shorter than 12.5 kb (ends of scaffolds and scaffolds shorter than that length) were
165 excluded from all analyses.

166

167 *Mitochondrial genome assembly and mtDNA analyses*

168 To reconstruct phylogenetic relationships between strains we reconstructed the entire mitochondrial
169 haplotype for strain G126 using MITObim (*de novo* approach of Hahn, Bachmann, & Chevreux, 2013)
170 with COII fragment downloaded from NCBI (accession KP168411.1) as an initial bait. We then
171 mapped reads from all sequenced strains to that sequence using bwa mem with default parameters.
172 Results were inspected manually using IGV. Duplicates were marked with picardtools and SNPs were
173 called using mpileup and bcftools assuming haploid samples. SNPs with quality lower than phred 30
174 were discarded from analyses. The nucleotide sequence for each strain was reconstructed with
175 bcftools consensus. Sequences were then aligned and used for phylogenetic analyses.

176 The evolutionary relationship between strains was inferred using ML with a GTR model, implemented
177 in MEGA X Software (Kumar et al., 2018). The same software and model were used to calculate mean
178 evolutionary distances between groups. BEAST2 (v2.6.1; Bouckaert et al., 2014) was then used to
179 perform Bayesian phylogenetic analysis of mitochondrial genomes and to estimate time to most
180 recent common ancestor (TMRCA). TMRCAs of nodes of interest were estimated based on
181 10,000,000 generations, logging every 1,000 steps, with the first 20% generations discarded as a
182 burn-in. We used a Yule Model as prior, HKY substitution model, and strict clock model. Divergence
183 time estimates were based on 13.1% divergence per million years estimated for *G. salaris* (see
184 Kuusela et al., 2007).

185

186 **Results**

187 A total of thirty *G. turnbulli* strains from three Trinidadian (Aripo, Caura and Lopinot Rivers) and three
188 Tobagonian populations (Dog River, Health Center and Spring Site; Figure 1A, details in
189 Supplementary Table 1) collected in two consecutive years were sequenced to an average coverage
190 of 38.7 per strain. Sequencing reads were then mapped to the reference nuclear genome and used
191 for SNP calling. We identified 776,881 single nucleotide- and 161,255 indel-polymorphisms. Principal
192 Component Analyses of SNP genotypes clearly separated Trinidad from Tobago, while differentiation
193 between local populations was present only in some cases (Figure 1B). In particular, strains from
194 Health Center and Dog River (Tobago) showed virtually no differentiation and, in contrast to the
195 Trinidadian populations, do not form monophyletic groups according to the populations of origin
196 (Figure 1C). The Trinidadian strains were more similar to the reference genome, obtained from a
197 strain on commercial guppies, *Gt3,* than strains collected from Tobago (Figure 1C). To remove the
198 potential effect of linkage on population differentiation, we pruned linked SNPs, but the general
199 pattern remained the same (Supplementary Figure 1). We calculated F_{ST} between Trinidad and
200 Tobago populations in 25 kb non-overlapping windows and found that 90% of the windows show F_{ST}
201 values between 0.25 and 0.55 (mean F_{ST} = 0.42; Supplementary Figure 2). Pairwise F_{ST} values were
202 higher among Trinidadian populations (Supplementary Figures 3) than among populations from
203 Tobago (Supplementary Figures 4). To resolve whether moderate F_{ST} between islands resulted from
204 moderate diversification between or from elevated levels of genetic variation within populations, we
205 calculated nucleotide diversity across Trinidad and Tobago strains separately. Nucleotide diversity on
206 Tobago was 2.4 times greater than that on Trinidad (0.0028 and 0.0012 respectively, Figure 1D). The
207 difference does not result from high differentiation among Tobagonian populations: nucleotide
208 diversity was also higher within Tobagonian populations, compared with those from Trinidad
209 (Supplementary Figure 5).

210 Increased nucleotide diversity on Tobago was mainly driven by high heterozygosity of most
211 individuals (Figures 1D-E). Similarly, inbreeding coefficient, F_{IS}, was negative for most of the strains

212 from Tobago, suggesting over-representation of heterozygous positions (Supplementary Figure 6). In
213 contrast, most of the Trinidadian strains showed positive values of F_{IS}.

214 High heterozygosity was observed in all strains from the Tobago populations Health Center and Dog
215 River. The third Tobago population (Spring Site) showed a mixture of patterns. One Spring Site strain
216 (G334) had low level of heterozygosity, reminiscent of the general pattern observed in Trinidadian
217 strains. Three other strains (G328, G367 and G368) showed bimodal distribution, with genomic
218 regions presenting either high or low level of heterozygosity. Finally, two Spring Site strains (G331
219 and G370) contained only highly heterozygous regions, similar to the pattern observed in Health
220 Center and Dog River (Figure 1E).

221 The majority of positions found polymorphic among Tobagonian strains (325,473 out of 498,111)
222 were heterozygous in all Health Center and Dog River strains, as well as and two (G331 and G370)
223 Spring Site strains. A total of 88% of these SNPs combined one allele from the reference genome and
224 the alternative allele found in the Tobagonian G334 strain (Figure 2). Genome-wide pattern of
225 divergence between re-sequenced strains and the reference genome demonstrated that strain G334
226 was the most divergent. Strains from Trinidad were consistently more closely related to the
227 reference genome (Figure 1C, Supplementary Figure 7). Based on these patterns we infer that highly
228 heterozygous Tobagonian strains consist of hybrids between two divergent haplotypes: one similar
229 to the reference sequence and the second similar to the G334 sequence. The same pattern with
230 shared heterozygous sites and high, genome-wide heterozygosity was observed in the Health Center
231 and Dog River strains from 2017 and 2018, indicating that the hybrid population is stable. This
232 implies an absence of recombination, as recombination would have fixed one of the two haplotypes
233 in at least some genomic regions. Auxiliary analysis comparing heterozygosity across all contigs to
234 that of a simulated, maximally heterozygous hybrid genome, created *in silico* from the reference
235 sequence and the G334 strain, confirmed that no such homozygous regions are observed in these
236 populations (Supplementary Figure 8). Furthermore, we compared genome wide nucleotide diversity

237 of Tobagonian strains collected in 2017 and 2018 and did not find evidence for a substantial
238 difference between time points (π_{2017} = 0.0018 vs π_{2018} = 0.0017). We also plotted relationships
239 between heterozygosity calculated in non-overlapping windows for each pair of strains and did not
240 find any indication for a reduction in heterozygosity in any strain (Supplementary Figure 9),
241 suggesting no ongoing recombination.

242 In contrast, divergent haplotypes have clearly recombined in the Spring Site population.
243 Three strains (G328, G367 and G368) show a mosaic pattern of the two haplotypes fixed in different
244 genomic locations, with other parts of the genome still remaining heterozygous (Figure 2,
245 Supplementary Figures 8 and 10). Signatures of sexual reproduction are also observed at least in
246 some of the strains from Trinidad. For example, SNPs localized in the scaffold scf7180000443747
247 (first sequence in the reference genome) show admixture of another haplotype in strain G314
248 coming from Lopinot, and another two strains in Lopinot are heterozygous in this region (Figure 2).
249 Another strain from the same population (G308) has negative F_{IS} (Supplementary Figure 6) and
250 slightly elevated genome-wide heterozygosity (Figure 1D), although the effect is weaker compared to
251 strains from Tobago. Nevertheless, heterozygous sites are on average rare in Trinidadian strains.

252 Clustering analyses (Figures 1B, 1C) and F_{ST} results (Supplementary Figure 4) based on nuclear SNPs
253 suggest low divergence between samples identified as hybrids. We explored it further by calculating
254 divergence in the haploid mitochondrial genomes. One Trinidadian strain (G126) was used for
255 mitochondrial genome assembly, and then reads from all other strains were mapped to that genome
256 for haplotype reconstruction. The resulting mt genome spans 14,159 bp. We found very little
257 divergence between strains identified as hybrids, compared to divergence amongst other samples
258 (Figure 3, Supplementary Figure 11). Putative hybrid strains from Health Center, Dog River and Spring
259 Site populations were found more closely related to Trinidadian strains than to remaining SPS strains,
260 confirming introgression inferred from genomic data. Within hybrids (Health Center, Dog River two
261 hybrid strains from Spring Site) the mean divergence between populations was 0.02%. In contrast,

262 the three other strains collected from Spring Site had strongly divergent mitochondria - the
263 nucleotide divergence between the two Spring Site groups was 1.58%. To independently verify low
264 divergence between Tobagonian hybrids and strains from Trinidad we sequenced a 262 bp fragment
265 of COII from 81 samples collected by us on Trinidad and Tobago. The results confirmed little
266 divergence between all Tobagonian samples except for some derived from the Spring Site population
267 (Supplementary Figure 12).

268 Lack of recombination in Tobago hampers identification of fast evolving regions in the genome,
269 because of strong linkage between sites. However, there was no evidence that the Trinidadian
270 populations experienced clonal expansion, and therefore rapidly evolving genes can be potentially
271 identified. For this reason, we tested for correlations between nucleotide diversity calculated in
272 windows separately for Tobagonian hybrid populations and for all Trinidadian strains. We used
273 nucleotide diversity calculated jointly for Health Center and Dog River strains as a proxy for the long-
274 term evolution of genomic regions (divergence between two haplotypes forming hybrids). Genomic
275 regions in which the nucleotide diversity calculated jointly for all strains from Trinidad deviates from
276 predictions based on this long-term evolutionary rate are therefore candidates for recent rapid
277 evolutionary change between years or populations. The divergence calculated in 25 kb windows
278 were highly correlated between Tobagonian hybrid populations and Trinidadian populations, while
279 few windows demonstrated elevated (according to overall trend) diversity in Trinidad (Figure 4A).
280 One scaffold (scf7180000445747; 19.4 kb long) showed extensive divergence between Trinidadian
281 strains collected in 2017 and 2018 (Figure 4B). It contains 7 protein coding genes, including putative
282 methylotransferase NSUN5, Endonuclease/exonuclease/phosphatase family domain-containing
283 protein 1, synaptogyrin and prohibitin.

284

285 **Discussion**

286 We examined whole genomes of thirty *G. turnbulli* strains, sampled from two Caribbean islands in
287 two consecutive years, providing insight into the evolution of their genomic diversity. On Tobago,
288 parasite populations tend to have much higher nucleotide diversity than those on Trinidad. This
289 higher diversity was caused by the presence of highly heterozygous strains in all studied Tobagonian
290 populations (50-100% of individuals per population). These strains shared most of the heterozygous
291 loci, with a repeatable pattern of having one reference-like allele and the other allele present in a
292 homozygous state only in one strain from Tobago (G334). This pattern led us to conclude that these
293 highly heterozygous strains comprise hybrids of two divergent lineages, one of which is close to a
294 strain isolated from ornamental guppies (Trinidad-like, used for the reference genome assembly),
295 and the second which is close to the Tobagonian G334 strain. In the Spring Site population (Tobago),
296 we observed two distinct mitochondrial lineages (diverged 145,000 years ago based on 13.1%
297 divergence per Mya, (Kuusela et al., 2007), 95% HPD: 133,900 – 155,700). Most likely they represent
298 mitochondrial genomes originating from two parental populations that formed Tobagonian hybrids.
299 The mitochondrial divergence between these two lineages was much larger than between
300 Tobagonian hybrids and Trinidadian populations (7,600 years, 95% HPD: 5,200 – 9,800 years),
301 demonstrating that hybrids inherited mitochondria from Trinidad-like ancestors. Initial hybridization
302 must have been followed by reproduction without recombination, otherwise recombination would
303 have produced both homozygous and heterozygous genotypes at least in some of the genomic
304 regions (as observed in two strains at Spring Site). In contrast, in most Tobagonian strains
305 heterozygosity was elevated genome-wide (Figure 1D), heterozygous genotypes were very similar for
306 all samples (Figure 2), suggesting that these genomes represent "frozen hybrids". Indeed, our
307 analyses imply that no recombination occurred since the hybrids were formed. If recombination
308 happened before expansion of the hybrid genotype, some regions with loss of heterozygosity would
309 be found and shared for all HC and DR strains. We did not find any evidence for loss of heterozygosity
310 in HC nor DR (Supplementary Figure 8). In contrast to HC and DR, patterns suggesting recombination
311 were obvious in some strains from the Spring Site population. Strains G328, G367 and G368 showed

312 non-identical patterns of heterozygosity across the nuclear genome, consistent with recent
313 independent outcrossing/selfing events followed by recombination. Two of these three Spring Site
314 strains (G328, G368) have a mitochondrial genome significantly divergent from other hybrids,
315 suggesting that subsequent rounds of outcrossing occurred between hybrid and non-hybrid
316 individuals, the latter carrying the divergent mtDNA. On Trinidad, a single individual showed slightly
317 elevated heterozygosity, possibly as a result of sexual reproduction between two moderately
318 diverged strains (G308, Figure 1D, Supplementary Figure 6), and indeed another individual from the
319 same population showed a pattern consistent with being a subsequent generation recombinant
320 (G312). Thus, in contrast to a set of pure hybrid populations we found on Tobago (Dog River and
321 Health Centre), recombination is not completely absent in other populations both on Tobago (Spring
322 Site) and on Trinidad. This evidence is consistent with the data from laboratory cross infections with
323 *G. turnbulli,* which demonstrated that 4-11% of individuals in mixed infections are hybrids with
324 ancestors from different strains used in the experiment (Schelkle et al., 2012).

325 Not only sexual reproduction, but also most forms of parthenogenesis involve meiotic
326 recombination, which is expected to lead to loss of heterozygosity spanning large genomic regions.
327 However, automictic parthenogenesis (automixis) with central fusion can freeze heterozygosity in
328 spite of recombination.(Engelstädter, 2017; Jaron, Bast, Ranallo-Benavidez, Robinson-Rechavi, &
329 Schwander, 2018) Under automixis with central fusion the probability of recombination increases
330 with distance from the centromere, and particularly regions distal to the centromere are affected if
331 recombination does occur. While, we are not able identify centromeres/telomeres in the reference
332 genome of *G. turnbulli*, we did not find any evidence for large genomic regions with loss of
333 heterozygosity (Supplementary Figures 11 and 12). Thus, either Tobagonian hybrids (i) reproduce
334 only mitotically, (ii) recombination is strongly suppressed during automictic parthenogenesis, or (iii)
335 recombinants are removed by selection leading to clonal-like evolution. Our data suggest that the
336 inferred, clonally-like reproducing hybrids spread relatively recently across Tobago, as indicated by
337 high similarity of their mitochondrial genomes, corresponding to 2,700 (95% HPD: 1,500 – 4,000)

338 years of divergence (Figure 3). The divergence time is based on the indirectly derived substitution
339 rate in a single mitochondrial locus of *Gyrodactylus salaris* (13.1% per Mya; Kuusela et al., 2007).
340 Other indirect estimates suggest lower (5.1% per Mya; Hahn et al., 2015) or higher substitution rates
341 (up to 20.3% per Mya; Meinilä et al., 2004) in *Gyrodactylus* species, and therefore divergence dating
342 should be taken with caution. Nevertheless, low nucleotide divergence between mitochondrial
343 genomes indicates a recent origin of the hybrids, and their widespread occurrence across Tobago
344 provides evidence for their evolutionary success. Hybrid genomes that were most widespread on
345 Tobago did not show any signs of recombination, and apparently remained stable, as would be
346 expected under predominantly clonal evolution.

347 What evolutionary forces are responsible for the apparent success of clonal hybrids on Tobago?
348 Tibayrenc and Ayala (2012, 2017) proposed that a predominance of clonal evolution is a common
349 adaptation to parasitism in micropathogens. Their hypothesis considers the total set of reproductive
350 strategies used by pathogens to escape 'recombination load'. In contrast, the 'epidemic clonality
351 model' (Smith, Smith, O'Rourke, & Spratt, 1993) advocates for the occurrence of occasional bouts of
352 clonally reproduced strains in otherwise recombining species. Ephemeral clones are frequently
353 replaced by other genotype combinations, maintaining a dominant role of sexual reproduction in
354 evolution. Complex patterns of outcrossing events in *G. turnbulli* populations seem to be consistent
355 with an 'epidemic clonality model' of evolution.

356 In the case of *G. turnbulli* a bout of clonality is associated with hybridization between two, relatively
357 divergent strains, confirming that hybridization of parasites can lead to the emergence of highly
358 successful, and potentially virulent strains. The potential to increase the virulence of *G. turnbulli* by
359 combining different genomes was suggested in an earlier report of laboratory experiments, in which
360 infection with two inbred stains led to higher parasite load compared to single strain infections
361 (Schelkle et al., 2012). However, the genomic divergence between these strains was unknown and, as

362 discussed by the authors, this result could be due to competition between clones leading to the
363 evolution of increased virulence (Schelkle et al., 2012).

364 Human activity, migrations and climate change shift the geographic distribution of many species
365 (Brooks & Hoberg, 2007; Cable et al., 2017; Lafferty, 2009), which increases the probability of
366 secondary contact between former distinct populations, potentially leading to hybridization followed
367 by adaptive evolution. Parasites are often transported with their hosts, and even if hosts do not
368 contribute to genetic pools of local populations, hitchhiking parasites can do so. This could be the
369 case for *G. turnbulli.* Guppies are commonly kept in aquaria, and *G. turnbulli* are regularly reported in
370 aquarium populations (Maceda-Veiga & Cable, 2019) from where they can potentially be released to
371 nature. Alternatively, hybrids might have appeared naturally. Our approximate dating of divergence
372 of mtDNA between hybrids from Trinidad and Tobago is consistent with the end of the last glacial
373 maximum, when a land bridge that probably existed between the islands during glaciation-related
374 sea level decline was disrupted again (H. J. Alexander, Taylor, Sze-Tsun Wu, & Breden, 2006;
375 Lambeck, 2004). This might have facilitated migration of guppies between the islands, and possibly
376 other fish species capable of spreading *G. turnbulli* (Cable et al., 2013; T. A. King & Cable, 2007).

377 Our recent study on the natural populations of another monogenean parasite infecting guppies
378 (*Gyrodactylus bullatarudis*) suggested that recent hybridization has also played a predominant role in
379 shaping genetic variation, but in that case in populations from Trinidad (Konczal et al., 2020).
380 Hybridization between two diverged lineages and its subsequent recombination resulted in a mosaic
381 genome composition of *G. bullatarudis*. Around half of the genome originates from one or another
382 lineage. High divergence between recombined lineages and relatively little divergence between local
383 populations led us to conclude that recent hybridization was followed by rapid expansion of a
384 recombinant *G. bulllatarudis* strain. Similar patterns of successful admixed hybrid genotypes have
385 been reported for many other parasites, including schistosomes (Kincaid-Smith et al., 2019; Platt et
386 al., 2019), *Trypanosoma* (see Tihon et al., 2017) and *Leishmania* (see Rogers et al., 2014) species, as

387 well as Dutch elm disease pathogens (Brasier, 2001; Hessenauer et al., 2020) and other fungi
388 pathogens (Mixão & Gabaldón, 2018) highlighting the role of recombination between divergent
389 genomes in parasite evolution. Our analysis of *G. turnbulli* genomes indicates, that unlike its
390 congener *G. bullatarudis,* natural expansion occurred without prior recombination. This scenario is
391 similar to suggestions for *Gyrodactylus salaris* – a significant pathogen of Atlantic salmon. The Baltic-
392 salmon specific *G. salaris* was proposed to have originated from a single hybridization event between
393 two clades, followed by the clonal reproduction of 'frozen hybrids', however this suggestion was
394 based on analyses of a single anonymous nuclear marker (Kuusela et al., 2007). Therefore, not only
395 clonal expansion of hybrids, but also linkage to lethal variants for both alleles (balanced lethal
396 system), or epistatic interactions could potentially explain the pattern observed in *G. salaris.* Our
397 genome-wide data suggest that clonal expansion of 'frozen hybrids' can indeed take place amongst
398 gyrodactylids.

399 Similar observations in other pathogens also suggest that 'frozen hybrids' might have a significant
400 evolutionary advantage. An interesting example comes from a common fungal parasite of
401 amphibians, *Batrachochytrium dendrobatidis,* in which asexually reproducing hybrids have higher
402 virulence than both parental strains in some host species (Farrer et al., 2011; Greenspan et al., 2018).
403 That hybrid is highly heterozygous and widespread. Most likely contact between previously
404 genetically isolated allopatric populations of the fungus facilitated hybridization, resulting in
405 generation, spread and invasion of the hypervirulent strain (Farrer et al., 2011). However, the strain
406 is not completely clonal and diversification can proceed by either mitotic or sexual recombination.
407 Similarly, in *Toxoplasma gondii* and *Sarcocystis neurona* outcrossing can precede a disease outbreak
408 (Grigg et al., 2001; Wendte et al., 2010), but subsequent selfing events were responsible for epidemic
409 expansions, and, as pointed out by the authors, it is not clear whether an out-cross is independently
410 sufficient to cause an epidemic (Wendte et al., 2010). Our data are therefore unique, in
411 demonstrating parasite expansion despite complete genome-wide suppression of recombination,

412 implying that 'frozen hybrids' might gain advantage from masking recessive, deleterious mutations or
413 from widening ecological niche.

414 Parasites may be particularly prone to accumulation of deleterious mutations in their genomes
415 (Criscione & Blouin, 2005). In general, the rate of accumulation depends primarily on the effective
416 population size. In small populations, random genetic drift can overpower selection making it easier
417 for deleterious mutations, particularly those with small effects, to become fixed (Kimura, Maruyama,
418 & Crow, 1963). This can cause mutational melt-down leading to population extinction (Michael Lynch
419 & Gabriel, 1990). Several features of macroparasite life cycles can act in concert to reduce effective
420 population sizes and increase the risk of such melt-down (Criscione & Blouin, 2005). In particular,
421 frequent bottlenecks associated with colonization/recolonization of new hosts can significantly
422 reduce the efficiency of selection in removing mildly deleterious mutations from populations.
423 Additionally, observations of *G. turnbulli* in the wild suggest that census population sizes are often
424 small and fluctuating over time (Stephenson, van Oosterhout, Mohammed, & Cable, 2015), which is
425 consistent with the low heterozygosity and generally positive inbreeding coefficient in the non-
426 hybrid populations that we investigated (Figure 1E, Supplementary Figure 6). If this leads to
427 homozygosity for a number of recessive or partially recessive mutations, hybridization between
428 divergent strains carrying those mutations at different genomic locations would result in hybrid vigor.
429 Alternatively, the advantage of hybridization can be due to alleles carried by heterozygotes being
430 adapted to different, and a wider range of, environments. In the case of parasites, divergent alleles
431 may each allow infection of different host species or genomes, allowing heterozygotes to infect a
432 wider range of hosts. For example, laboratory experiments on tapeworms in sticklebacks showed
433 that two genetic lines were only able to infect a single respective host species, whereas their hybrids
434 could infect both species (Henrich, Benesh, & Kalbe, 2013). In the case of *G. turnbulli,* however,
435 hybridization occurred among two strains infecting the same host species.

436 Clonal expansion of hybrids is likely facilitated by facultative sexual reproduction of *Gyrodactylus*
437 species. In such systems, switching between facultative and obligate clonal reproduction does not
438 appear to be evolutionarily constrained and can easily evolve when favoured by selection (Neiman et
439 al., 2014). However, though it has been proposed that hybridization is the main mechanism
440 responsible for evolution of asexual reproduction, its causality and underlying mechanisms are
441 poorly understood (Janko et al., 2018; Neiman et al., 2014; Schön, van Dijk, & Martens, 2009; Xu,
442 Innes, Lynch, & Cristescu, 2013). Mortiz et al. (1989) suggested that reproducing asexually results
443 from the accumulation of incompatibilities between hybridizing species that disrupts cellular
444 regulation of sexual reproduction. In fact, the majority of species which recently evolved asexual
445 reproduction are of hybrid origin, although it concerns primarily vertebrates and it is not clear
446 whether it applies also to other taxa (Neaves & Baumann, 2011). In the case of *G. turnbulli,* however,
447 recombination is not completely suppressed, because it was observed in one of the hybrid
448 populations on Tobago (Spring Site), where it might have occurred between hybrid and non-hybrid
449 individuals. The fact that recombinant individuals are rare on Tobago might result either from rarity
450 of recombination events, or from effective removal of recombinants from the population by
451 selection. The disadvantage of recombinants may stem from restoring homozygotes for recessive
452 deleterious mutations. Discrimination between these two possibilities ideally requires direct
453 estimates of recombination rate, possibly based on changes in genotype frequencies over time in
454 both hybrid and non-hybrid populations (Becheler et al., 2017).

455 Recombination can, at least to some degree, enable independent evolution of different genomic
456 regions, allowing identification of genomic regions with elevated genomic variation between the two
457 sampling years, possibly driven by selection. We therefore searched for such regions in Trinidad
458 populations and found that one genomic region showed an extreme pattern of genetic diversity, with
459 several interesting genes previously associated with modulating lifespan, stress resistance
460 (Methylotrasferase NSUN5; Schosserer et al., 2015) and host-parasite interactions (Prohibitin; Jain et

al., 2010). Future work including more individuals and experimental validation is needed to confirm involvement of these genes in coevolutionary dynamics.

Concluding, our population genomic dataset of *G. turnbulli* allowed us to infer that hybridization followed by clonal reproduction contributed to evolutionary success of these parasites on Tobago. It is likely that the evolutionary success stems from heterotic effects, which are frozen in hybrids. Benefits of such freezing may only be temporary, as the lack of recombination may hinder the pathogen in keeping up in the evolutionary arms-race with the host's immune system. That recombination seemed to be maintained in Trinidadian populations suggests that it may be favoured in the long run as well. It would be interesting to see if after initial success, recombination would again be favoured, as might have been the case with *G. bullatarudis* (see Konczal et al. 2020). Our results highlight that a single outcrossing event is independently sufficient to cause pathogen expansion, thus emphasizing that such processes can result in emergence of invasive pathogens, some of which may be of public health and conservation concern.

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Data accessibility

The raw sequences are available as FASTQ files in the GeneBank (BioProject accession no. PRJNA603444).

Author Contributions

M.K. and J.R. designed research; M.K., K.J.P. and R.S.M. collected samples; C.H. provided reference genome; M.K. analysed data with suggestions from J.R., J.C. and C.H.; MK drafted manuscript, J.R. and J.C. contributed to manuscript writing. All authors corrected manuscript and approved the final version.

Figure captions

Figure 1 Patterns of genetic variation in *Gyrodactylus turnbulli* populations from Trinidad and Tobago. A: Map of Trinidad and Tobago with sampling sites. **B:** Principal Component Analyses of *G. turnbulli* strains. PCA based on 673,104 SNPs which passed filtering criteria. SNPs were identified within 30 isolates from three Trinidadian and three Tobagonian populations. **C:** Neighbor Joining tree (Saitou and Nei 1987) constructed using distance matrix, with one minus identity-by-state units, calculated based on 673,104 SNPs. Populations are represented by different colours. The reference sequence (obtained from a strain on commercial guppies) is shown in black. **D:** Distribution of nucleotide diversity (π) calculated across the genome in 25 kb non-overlapping windows. **E:** Distribution of heterozygosity calculated in 25 kb non-overlapping windows for each strain collected from Trinidad (white background) and Tobago (shaded background) populations.

Figure 2. Graphical representation of genotypes in *Gyrodactylus turnbulli* strains. Genotypes identified as reference homozygotes are shown in green, alternative homozygotes in red, heterozygous in yellow. Strains collected in 2017 and 2018 are labeled in grey and black respectively, and those collected from different local populations are separated by horizontal grey lines. A subset of polymorphic sites (10,000 SNPs) from the longest scaffolds is shown.

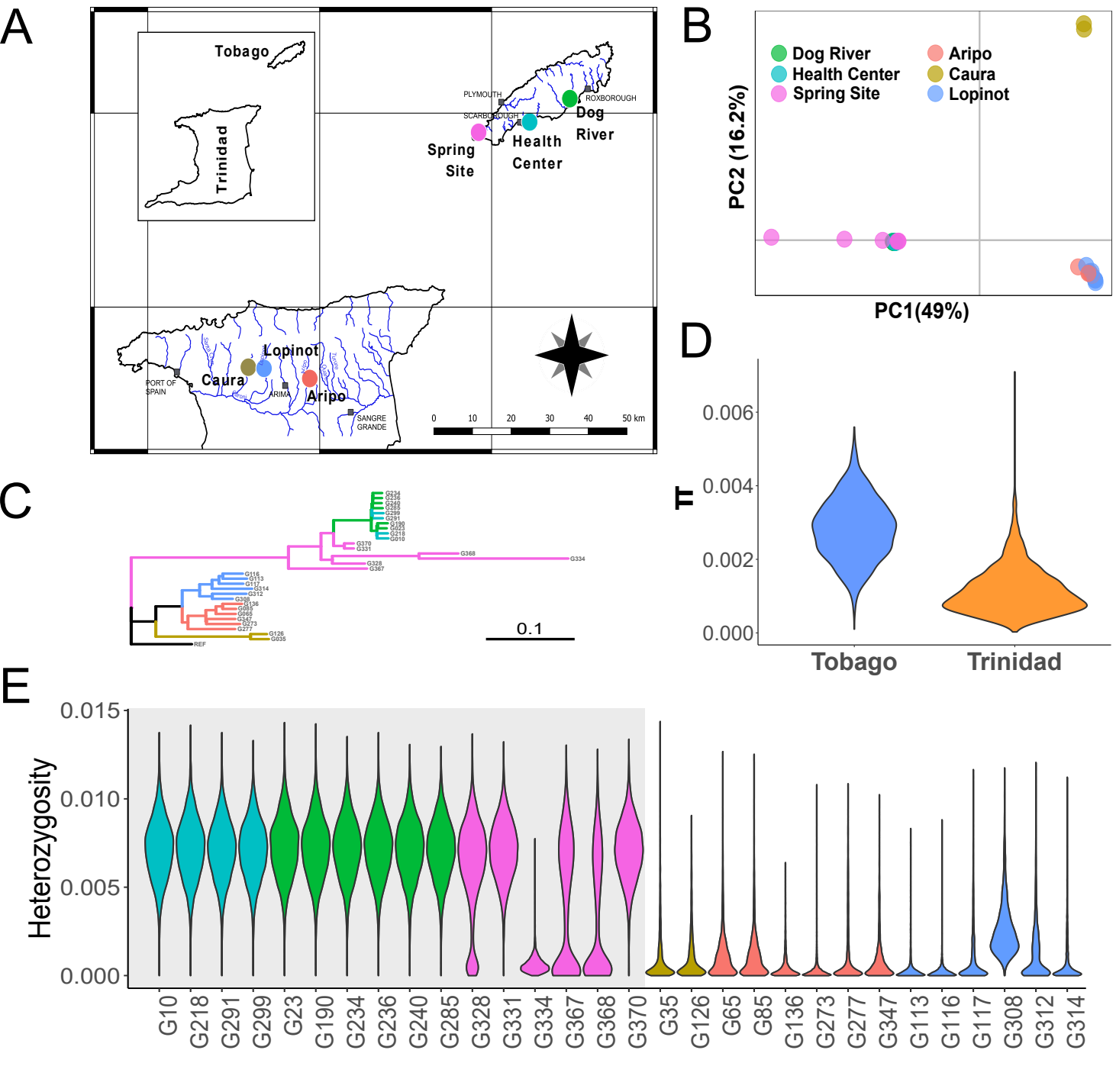
Figure 3. Phylogenetic relationship between *Gyrodactylus turnbulli* strains, reconstructed based on mitochondrial genome sequences (14,159 bp). The trees were derived via a Bayesian phylogenetic method, using 10,000,000 generations of MCMC samples, with HKY substitution model and strict

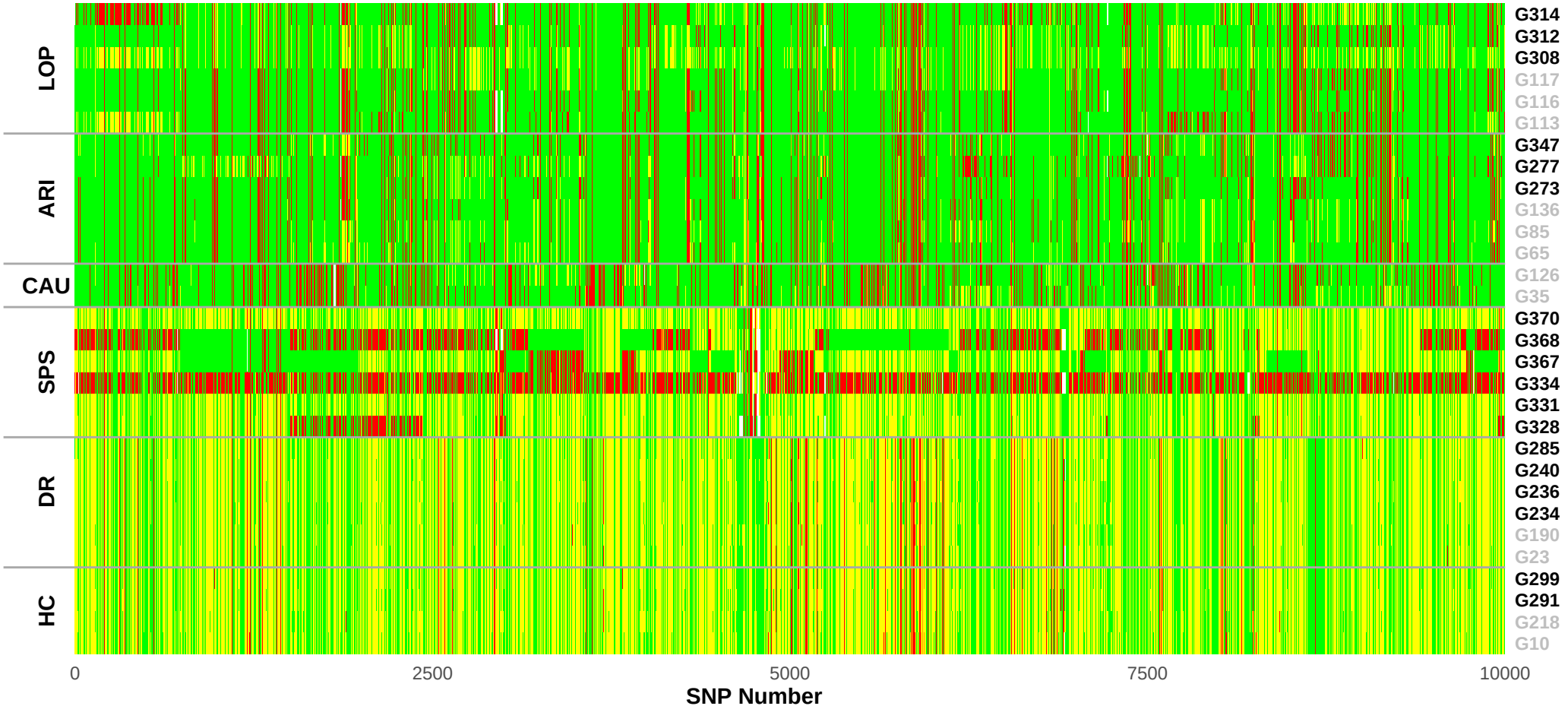
734 clock model, assuming 13.1% divergence per million years. The estimated time before present (in
735 years) for tree nodes is shown at the bottom of the tree.

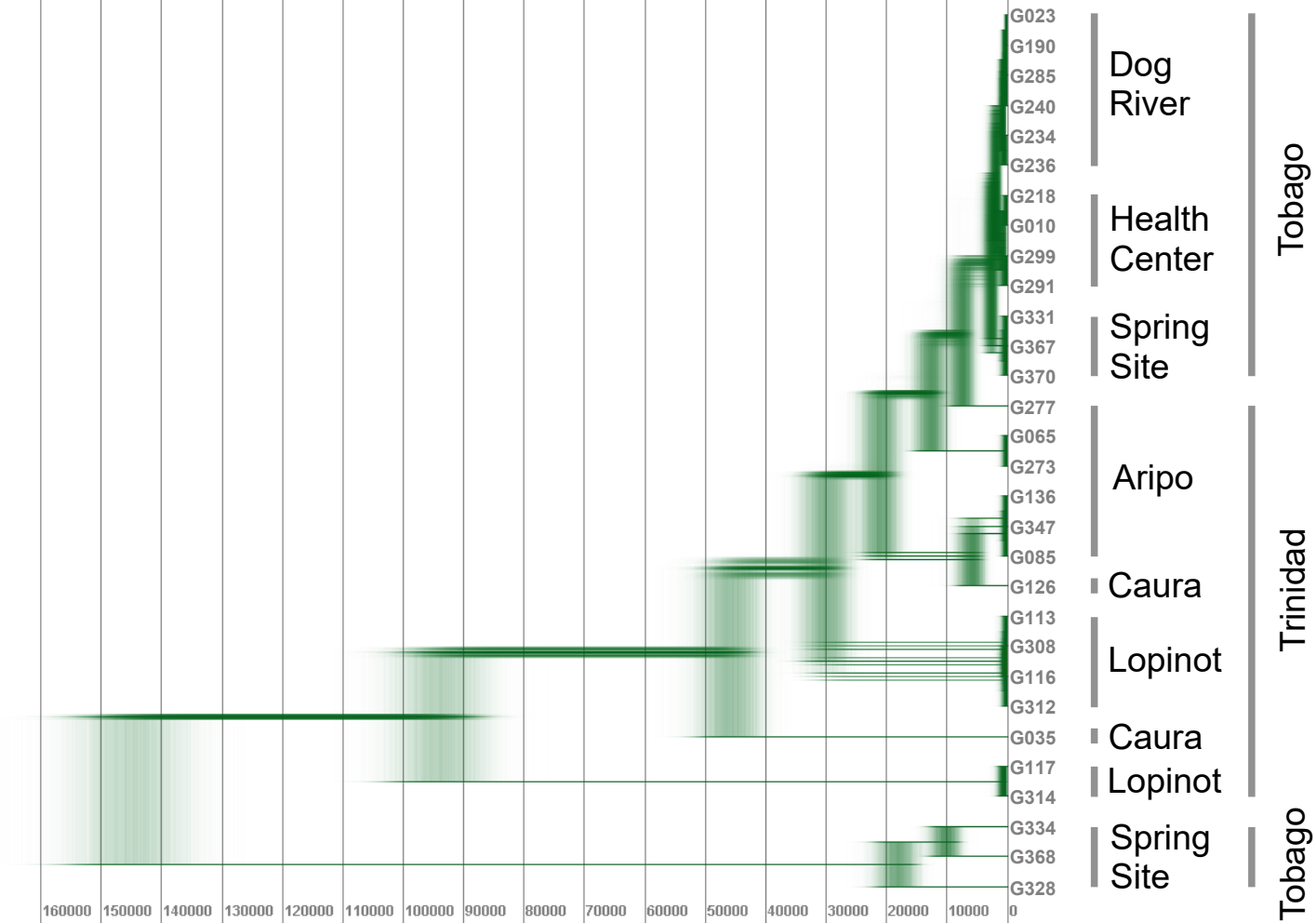
736

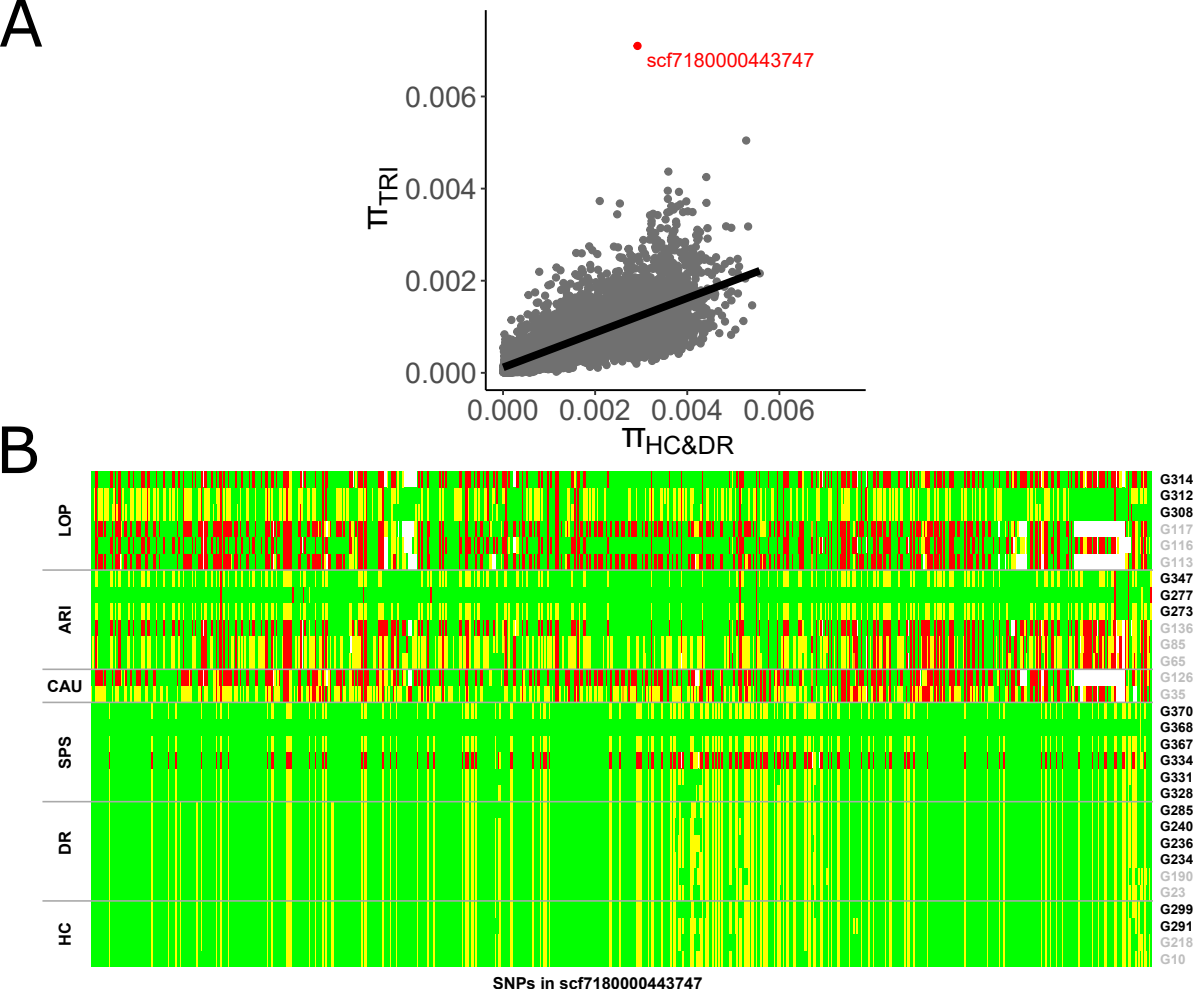
737 **Figure 4 Genetic variation in genomic region with elevated nucleotide diversity in populations from**
738 **Trinidad. A:** Correlation between nucleotide diversity calculated jointly for Health Center and Dog
739 River populations and nucleotide diversity calculated jointly for all strains from Trinidad. Black line
740 represents linear regression (y = 0.00 + 0.38*x). Scaffold scf7180000443747 is shown in red. **B:**
741 Graphical representation of genotypes in polymorphic sites of *Gyrodactylus turnbulli* localized in
742 scaffold scf7180000443747 (707 SNPs). Genotypes identified as reference homozygotes are shown in
743 green, alternative homozygotes in red, heterozygous in yellow. Strains collected in 2017 and 2018
744 are shown in grey and black respectively, and those collected from different local populations are
745 separated by horizontal grey lines.

746


A
Tobago
Trinidad
PLYMOUTH
SCARBOROUGH
ROXBOROUGH
Dog River
Health Center
Spring Site
Lopinot
Caura
Aripo
PORT OF SPAIN
ARIMA
SANGRE GRANDE
0 10 20 30 40 50 km
B
Dog River
Health Center
Spring Site
Aripo
Caura
Lopinot
PC2 (16.2%)
PC1(49%)
C
0.1
D
0.006
0.004
0.002
0.000
Tobago
Trinidad
E
Heterozygosity
0.015
0.010
0.005
0.000
G10
G218
G291
G299
G23
G190
G234
G236
G240
G285
G328
G331
G334
G367
G368
G370
G35
G126
G65
G85
G136
G273
G277
G347
G113
G116
G117
G308
G312
G314


LOP
ARI
CAU
SPS
DR
HC
G314
G312
G308
G117
G116
G113
G347
G277
G273
G136
G85
G65
G126
G35
G370
G368
G367
G334
G331
G328
G285
G240
G236
G234
G190
G23
G299
G291
G218
G10
0
2500
5000
7500
10000
SNP Number


G023
G190
G285
G240
G234
G236
G218
G010
G299
G291
G331
G367
G370
G277
G065
G273
G136
G347
G085
G126
G113
G308
G116
G312
G035
G117
G314
G334
G368
G328
Dog River
Health Center
Spring Site
Aripo
Caura
Lopinot
Caura
Lopinot
Spring Site
Tobago
Trinidad
Tobago
160000
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0


A
scf7180000443747
0.006
0.004
0.002
0.000
π_{TRI}
0.000
0.002
0.004
0.006
$\pi_{HC\&DR}$
B
LOP
ARI
CAU
SPS
DR
HC
G314
G312
G308
G117
G116
G113
G347
G277
G273
G136
G85
G65
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G35
G370
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G285
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G236
G234
G190
G23
G299
G291
G218
G10
SNPs in scf7180000443747