




# 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 vigour 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 resequenced 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 with native genotypes. Our results thus highlight that a single outcrossing event may be independently sufficient to cause pathogen expansion.

## KEYWORDS

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

## 1 | 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 et al., 2015). This role is

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

Parasite strains of hybrid origin can gain an advantage because recombinants—individuals with shuffled genomes—produced after hybridization may show enhanced phenotypic characteristics compared with the parental generation, such as higher infectivity (Grigg et al., 2001), expanded host range (Detwiler & Criscione, 2010), increased transmission potential (Volf et al., 2007) or

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increased tolerance to harsh environmental conditions (Laine et al., 2019). Hybridization, however, is often associated with asexual reproduction, which may in the long term preclude the initial benefits resulting from recombination between divergent genomes, since asexual lineages tend to be less adaptable to environmental change compared with sexually reproducing lineages (Neaves & Baumann, 2011).

Conversely, asexual reproduction may be selectively beneficial if clonal reproduction 'freezes' heterozygosity that confers short-term evolutionary advantage (Neiman et al., 2014). F1 hybrids often show greater vigour, productivity, disease resistance or overall increased Darwinian fitness (Kingsbury, 2009). Two alternative hypotheses may explain the existence of hybrid vigour (heterosis) (Lippman & Zamir, 2007). The overdominance hypothesis attributes heterosis to superior fitness of heterozygous genotypes over homozygous wild types. The dominance hypothesis assumes that recessive deleterious mutations are to some extent masked in a heterozygous stage. Recent theoretical work indicates that dominance-induced heterosis might play an important role in evolution, especially if deleterious alleles are not effectively removed from populations as it is expected when populations are small (Gilbert et al., 2020; Kim et al., 2018; Zhao & Charlesworth, 2016). This mechanism may be particularly relevant to parasite evolution. Many parasite populations are small or experience frequent bottlenecks, therefore are prone not only to the accumulation of deleterious mutations (Lynch et al., 1993), but also to elevated homozygosity due to inbreeding. In such a case, one could expect higher fitness of hybrid parasites. Successful parasite strains originating from a hybridization event have been documented in several species, including *Batrachochytrium dendrobatidis* (see Farrer et al., 2011; Greenspan et al., 2018), *Toxoplasma gondii* and *Sarcocystis neurona* (see Grigg et al., 2001; Wendte et al., 2010). However, because in all these cases some degree of recombination occurred, the relative role of heterozygosity versus recombination between highly divergent genomes remains unresolved.

Here, we report frozen genome-wide heterozygosity in wild *Gyrodactylus turnbulli*, a common ectoparasite of the guppy (*Poecilia reticulata*) and well-known model species in epidemiological and eco-evolutionary research (e.g. Houde & Torio, 1992; Jacquin et al., 2016; van Oosterhout et al., 2003; Phillips et al., 2018; Reynolds et al., 2018). Previous laboratory work demonstrated that *G. turnbulli* from mixed strain infections often hybridize and have higher fitness (more parasites per host, longer infection, but not increased host mortality), suggesting that in fact hybrid individuals might have increased fitness, possibly due to heterosis or recombination (Schelkle et al., 2012). However, the significance of hybridization in wild populations is unknown (Xavier et al., 2015). We analyse 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 highlighting that a single hybridization event can lead to the emergence of highly successful parasites, despite suppressed recombination.

## 2 | MATERIALS AND METHODS

### 2.1 | 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] and Lopinot River [LOP]; Tobago: Dog River [DR], Health Center [HC] and Spring Site [SPS]). All Trinidadian sites are located in the Caroni drainage, while Tobagonian locations are not interconnected. Fish were transported to our field station in Charlottesville, 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 dissecting 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 (tricaine methanesulfonate) 8–13 days postinfection. The number of worms was counted, and samples were preserved in 97% molecular grade ethanol and transported to Poland. Samples with at least 10 worms were used for DNA extraction and for species identification as described below. Parasites collected in 2017 and identified as *G. bullatarudis* were reported in a separate study (Konczal et al., 2020), while this work focused solely on *G. turnbulli*.

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- to 2.5-day 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 et al., 2007; Cable & Harris, 2002). Even though it is unclear which mechanism restores the diploid component during parthenogenesis (Cable & Harris, 2002), it has been suggested that heterozygosity is maintained during that process, as reported for *G. salaris* (see Kuusela et al., 2007). Third-born and subsequent born daughters may result from outcrossing, selfing or parthenogenesis (Cable & Harris, 2002). Given our experimental design, we expect that the majority of gyrodactylid individuals on each experimentally infected fish developed from first or second births and should have preserved founders' genomes.

### 2.2 | DNA extraction, library preparation and sequencing

DNA was extracted from pools of individuals ('strain' henceforth) derived from a single worm collected from a single experimentally

infected fish using MagJET Genomic DNA Kits (Thermo Scientific). DNA concentration was measured using Qubit High-Sensitivity reagents. *Gyrodactylus* were identified to species using the Sanger sequencing of a 262-bp fragment of the mitochondrial protein-coding COII gene (Xavier et al., 2015). Sequences were aligned together with records downloaded from the NCBI GenBank, and a neighbour-joining tree was reconstructed with MEGA X (Kumar et al., 2018) to determine species. Thirty strains were selected for library preparation (Nextera DNA Flex Library Prep Kit, Illumina) based on DNA quantity and population of origin. Subsets of strains were sequenced in 2018 with HiSeq 2500 (2 x 100 bp). All other strains were sequenced in 2019 on the NovaSeq 6000 platform in 2 x 150 bp mode (see Table S1 for more details).

### 2.3 | SNP calling and population genomic analyses

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

We then used SNPs to calculate PCA with plink (version 1.90, Purcell et al., 2007) using only SNPs with minor allele frequency greater than 0.05. The same data set with additional sample representing reference genotype (all genotypes encoded as reference homozygous) was used to calculate distance matrix (in one minus identity-by-state units) with plink and to construct a neighbour-joining tree using the ape package in R. In addition, we ran PCAs with SNPs that were previously pruned for linkage disequilibrium. We first pruned SNPs with -indep-pairwise 100 10 0.1 option in plink and then used the same program to calculate PCA.

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

it by a window length. Windows shorter than 12.5 kb (ends of scaffolds and scaffolds shorter than that length) were excluded from all analyses.

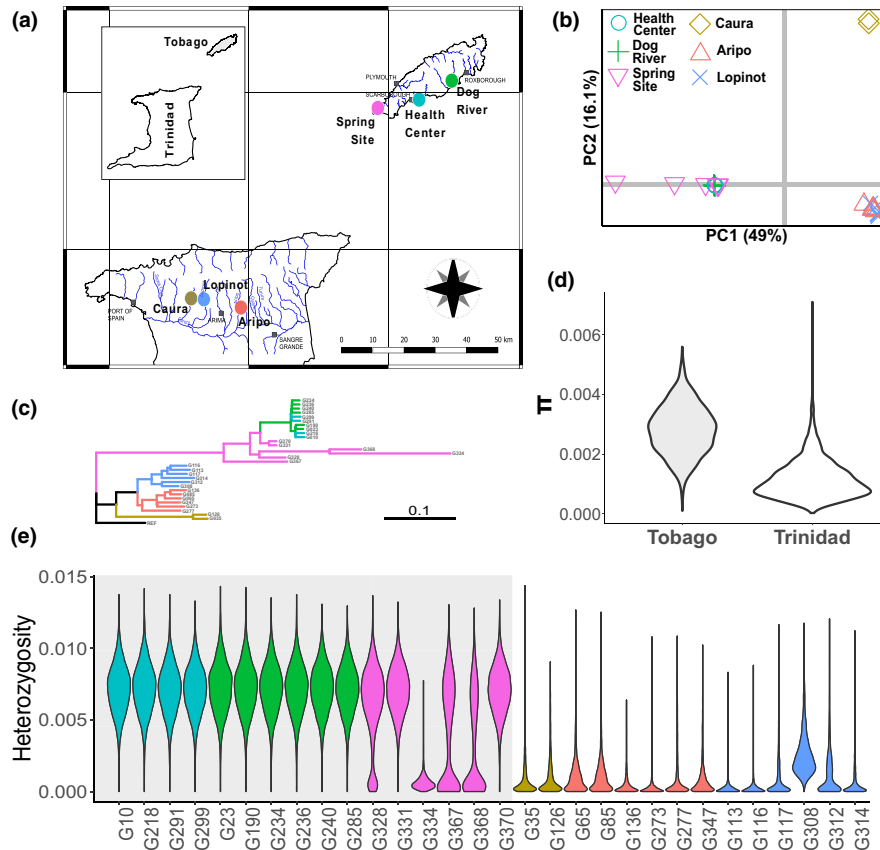
### 2.4 | Mitochondrial genome assembly and mtDNA analyses

To reconstruct phylogenetic relationships between strains, we reconstructed the entire mitochondrial haplotype for strain G126 using MITObim (de novo approach of Hahn et al., 2013) with COII fragment downloaded from NCBI (Accession No. KP168411.1) as an initial bait. We then mapped reads from all sequenced strains to that sequence using bwa mem with default parameters. Results were inspected manually using IGV. Duplicates were marked with picardtools, and SNPs were called using mpileup and bcftools assuming haploid samples. SNPs with quality lower than phred 30 were discarded from analyses. The nucleotide sequence for each strain was reconstructed with bcftools consensus. Sequences were then aligned and used for phylogenetic analyses.

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

## 3 | RESULTS

A total of thirty *G. turnbulli* strains from three Trinidadian (Aripo, Caura and Lopinot rivers) and three Tobagonian populations (Dog River, Health Center and Spring Site; Figure 1A, details in Table S1) collected in two consecutive years were sequenced to an average coverage of 38.7 per strain. Sequencing reads were then mapped to the reference nuclear genome and used for SNP calling. We identified 776,881 single nucleotide and 161,255 indel polymorphisms. Principal component analyses of SNP genotypes clearly separated Trinidad from Tobago, while differentiation between local populations was present only in some cases (Figure 1B). In particular, strains from Health Center and Dog River (Tobago) showed virtually no differentiation and, in contrast to the Trinidadian populations, did not form monophyletic groups according to the populations of origin (Figure 1C). The Trinidadian strains were more similar to the reference genome, obtained from a strain on commercial guppies, Gt3, than strains collected from Tobago (Figure 1C). To remove the



**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) Neighbour-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 colour-coded as in (A). The reference sequence (obtained from a strain on commercial guppies) is shown in black. (D) Distribution of nucleotide diversity ( $\pi$ ) calculated across the genome in 25-kb nonoverlapping windows. (E) Distribution of heterozygosity calculated in 25-kb nonoverlapping windows for each strain collected from Trinidad (white background) and Tobago (shaded background) populations [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

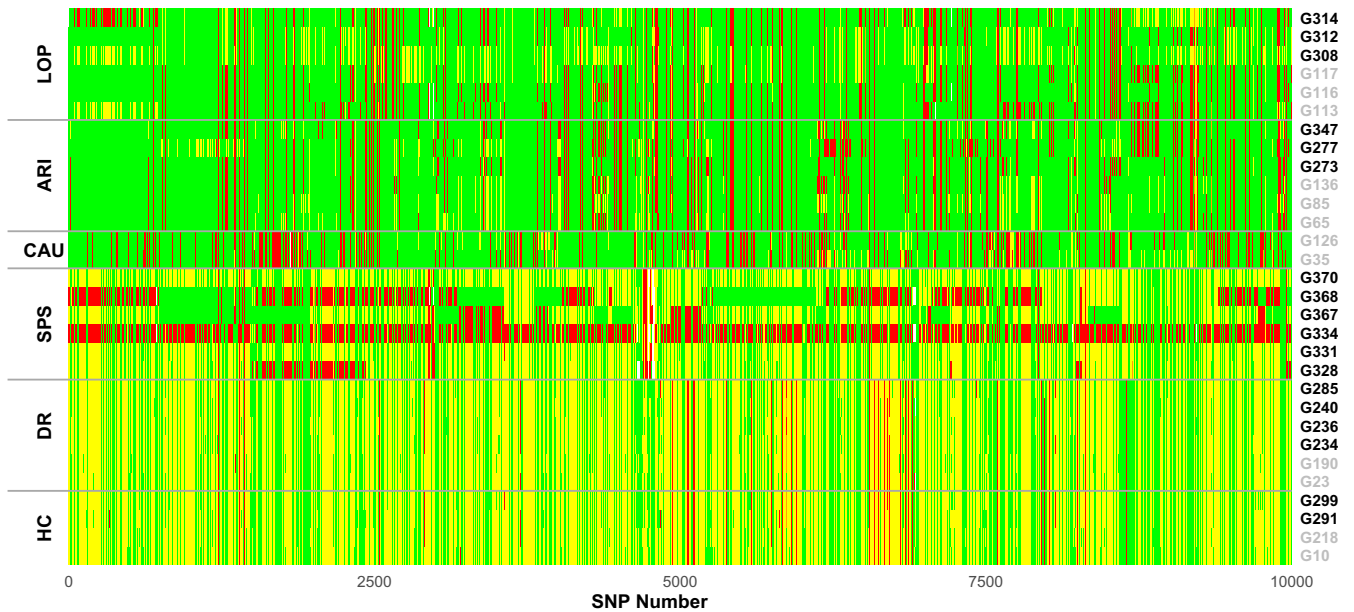
potential effect of linkage on population differentiation, we pruned linked SNPs, but the general pattern remained the same (Figure S1). We calculated  $F_{ST}$  between Trinidad and Tobago populations in 25-kb nonoverlapping windows and found that 90% of the windows show  $F_{ST}$  values between 0.25 and 0.55 (mean  $F_{ST}$  = 0.42; Figure S2). Pairwise  $F_{ST}$  values were higher among Trinidadian populations (Figures S3) than among populations from Tobago (Figures S4). To resolve whether moderate  $F_{ST}$  between islands resulted from moderate diversification between or from elevated levels of genetic variation within populations, we calculated nucleotide diversity across Trinidad and Tobago strains separately. Nucleotide diversity on Tobago was 2.4 times greater than that on Trinidad (0.0028 and 0.0012, respectively, Figure 1D). The difference does not result from high differentiation among Tobagonian populations: nucleotide diversity was also higher within Tobagonian populations, compared with those from Trinidad (Figure S5).

Increased nucleotide diversity on Tobago was mainly driven by high heterozygosity of most individuals (Figure 1D-E). Similarly,

inbreeding coefficient,  $F_{IS}$ , was negative for most of the strains from Tobago, suggesting over-representation of heterozygous positions (Figure S6). In contrast, most of the Trinidadian strains showed positive values of  $F_{IS}$ .

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

The majority of positions found polymorphic among Tobagonian strains (325,473 out of 498,111) were heterozygous in all Health Center and Dog River strains, as well as two (G331 and G370) Spring Site strains. A total of 88% of these SNPs combined



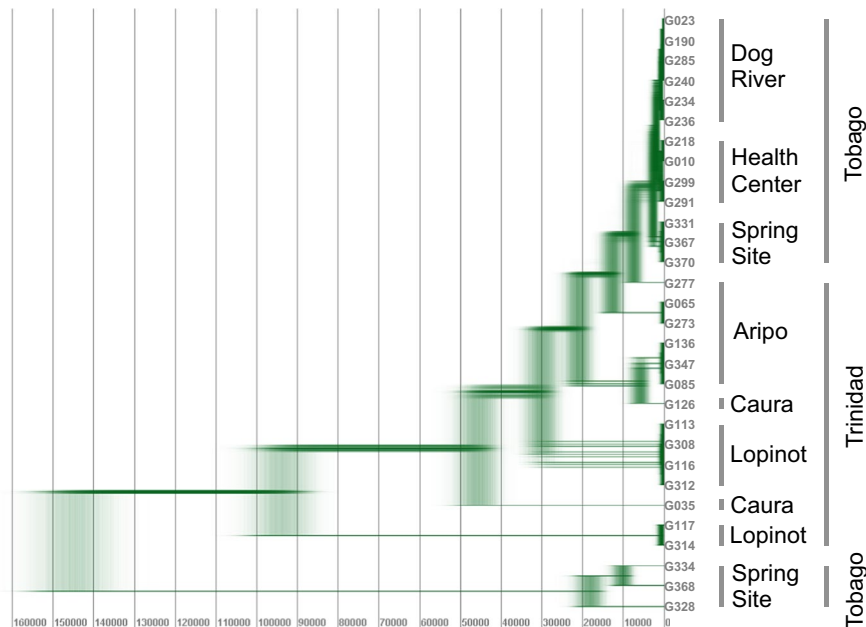
**FIGURE 2** Graphical representation of genotypes in *Gyrodactylus turnbulli* strains. Genotypes identified as reference homozygotes are shown in green, alternative homozygotes in red and heterozygous in yellow. Strains collected in 2017 and 2018 are labelled in grey and black, respectively, and those 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 [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

one allele from the reference genome and the alternative allele found in the Tobagonian G334 strain (Figure 2). The genome-wide pattern of divergence between resequenced strains and the reference genome demonstrated that strain G334 was the most divergent. Strains from Trinidad were consistently more closely related to the reference genome (Figure 1C, Figure S7). Based on these patterns, we infer that highly heterozygous Tobagonian strains consist of hybrids between two divergent haplotypes: one similar to the reference sequence and the second similar to the G334 sequence. The same pattern with shared heterozygous sites and high, genome-wide heterozygosity was observed in the Health Center and Dog River strains from 2017 and 2018, indicating that the hybrid population is stable. This implies an absence of recombination, as recombination would have fixed one of the two haplotypes in at least some genomic regions. Auxiliary analysis comparing heterozygosity across all contigs to that of a simulated, maximally heterozygous hybrid genome, created in silico from the reference sequence and the G334 strain, confirmed that no such homozygous regions are observed in these populations (Figure S8). Furthermore, we compared genome-wide nucleotide diversity of Tobagonian strains collected in 2017 and 2018 and did not find evidence for a substantial difference between time points ( $\pi_{2017} = 0.0018$  vs  $\pi_{2018} = 0.0017$ ). We also plotted relationships between heterozygosity calculated in nonoverlapping windows for each pair of strains and did not find any indication for a reduction in heterozygosity in any strain (Figure S9), suggesting no ongoing recombination.

In contrast, divergent haplotypes have clearly recombined in the Spring Site population. Three strains (G328, G367 and G368) show

a mosaic pattern of the two haplotypes fixed in different genomic locations, with other parts of the genome still remaining heterozygous (Figure 2, Figures S8 and S10). Signatures of sexual reproduction were also observed at least in some of the strains from Trinidad. For example, SNPs localized in the scaffold scf7180000443747 (first sequence in the reference genome) show admixture of another haplotype in strain G314 coming from Lopinot, and another two strains in Lopinot are heterozygous in this region (Figure 2). Another strain from the same population (G308) has negative  $F_{IS}$  (Figure S6) and slightly elevated genome-wide heterozygosity (Figure 1D), although the effect is weaker compared with strains from Tobago. Nevertheless, heterozygous sites are on average rare in Trinidadian strains.

Clustering analyses (Figure 1B, 1C) and  $F_{ST}$  results (Figure S4) based on nuclear SNPs suggest low divergence between samples identified as hybrids. We explored it further by calculating divergence in the haploid mitochondrial genomes. One Trinidadian strain (G126) was used for mitochondrial genome assembly, and then, reads from all other strains were mapped to that genome for haplotype reconstruction. The resulting mt genome spans 14,159 bp. We found very little divergence between strains identified as hybrids, compared with divergence among other samples (Figure 3). Putative hybrid strains from Health Center, Dog River and Spring Site populations were more closely related to Trinidadian strains than to remaining SPS strains, confirming introgression inferred from genomic data. Within hybrids (Health Center, Dog River and two hybrid strains from Spring Site), the mean divergence between populations was 0.02%. In contrast, the three other strains collected from Spring Site had strongly divergent mitochondria—the nucleotide divergence



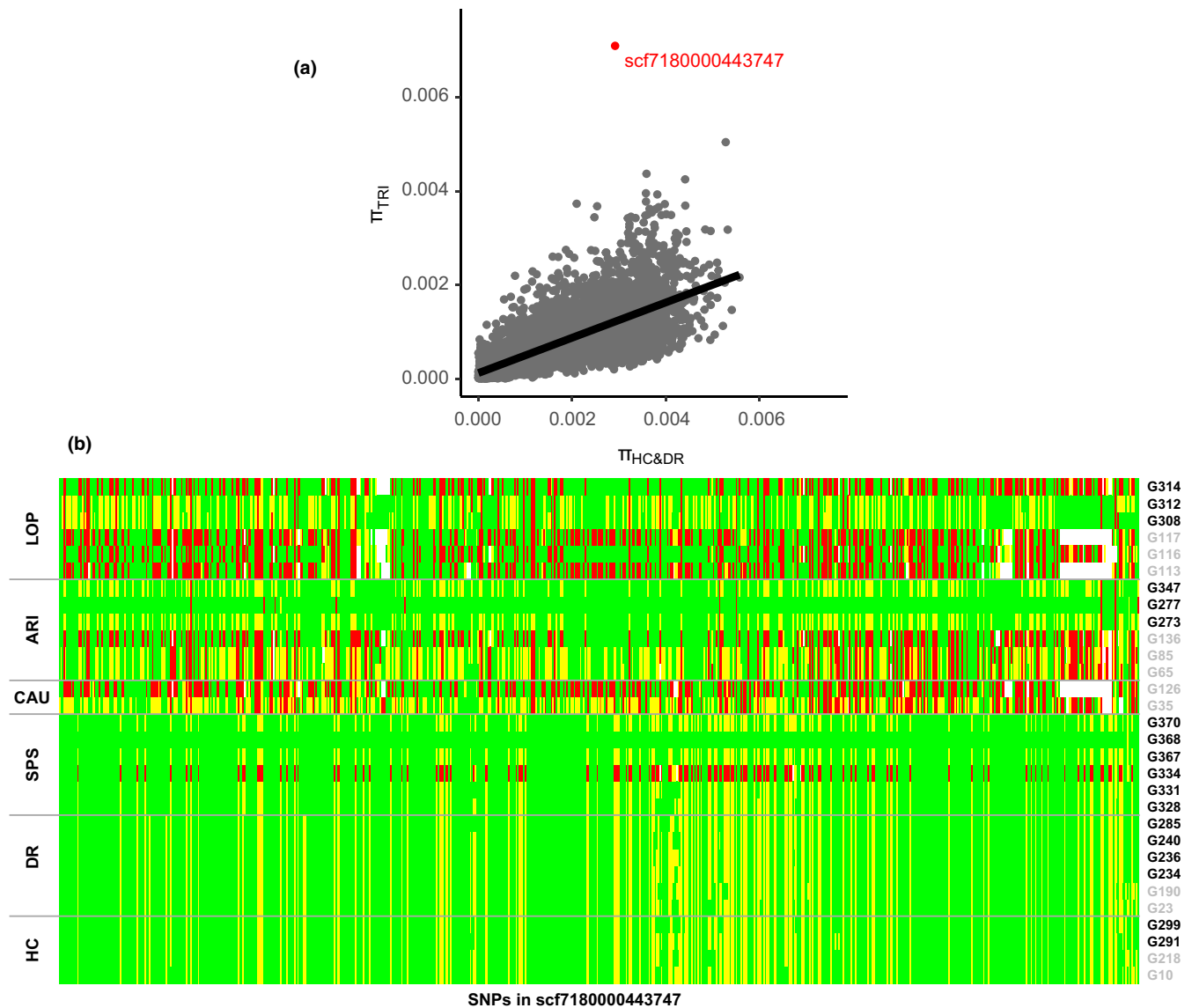
**FIGURE 3** Phylogenetic relationship between *Gyrodactylus turnbulli* strains, reconstructed based on mitochondrial genome sequences (14,159 bp). The trees were derived via a Bayesian phylogenetic method, using 10,000,000 generations of MCMC samples, with HKY substitution model and strict clock model, assuming 13.1% divergence per million years. The estimated time before present (in years) for tree nodes is shown at the bottom of the tree [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

between the two Spring Site groups was 1.58%. To independently verify low divergence between Tobagonian hybrids and strains from Trinidad, we sequenced a 262-bp fragment of COII from 81 samples collected by us on Trinidad and Tobago. The results confirmed little divergence between all Tobagonian samples except for some derived from the Spring Site population (Figure S11).

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

## 4 | DISCUSSION

We examined whole genomes of thirty *G. turnbulli* strains, sampled from two Caribbean islands in two consecutive years, providing insight into the evolution of their genomic diversity. On Tobago, parasite populations tend to have much higher nucleotide diversity than those on Trinidad. This higher diversity was caused by the presence of highly heterozygous strains in all studied Tobagonian populations (50–100% of individuals per population). These strains shared most of the heterozygous loci, with a repeatable pattern of having one reference-like allele and the other allele present in a homozygous state only in one strain from Tobago (G334). This pattern led us to conclude that these highly heterozygous strains comprise hybrids of two divergent lineages, one of which is close to a strain isolated from ornamental guppies (Trinidad-like, used for the reference genome assembly), and the second which is close to the Tobagonian G334 strain. In the Spring Site population (Tobago), we observed two distinct mitochondrial lineages (diverged 145,000 years ago based on 13.1% divergence per Mya (Kuusela et al., 2007), 95% HPD: 133,900 – 155,700). Most likely they represent mitochondrial genomes originating from two parental populations that formed Tobagonian hybrids. The mitochondrial divergence between these two lineages was much larger than between Tobagonian hybrids and Trinidadian populations (7,600 years, 95% HPD: 5,200–9,800 years), demonstrating that hybrids inherited mitochondria from Trinidad-like ancestors. Initial hybridization must have been followed by reproduction without recombination, otherwise recombination would have produced both homozygous and heterozygous genotypes at least in some of the genomic regions (as observed in two strains at



**FIGURE 4** Genetic variation in genomic region with elevated nucleotide diversity in populations from Trinidad. (A) Correlation between nucleotide diversity calculated jointly for Health Center and Dog River populations and nucleotide diversity calculated jointly for all strains from Trinidad. Black line represents linear regression ( $y = 0.00 + 0.38 \cdot x$ ). Scaffold scf7180000443747 is shown in red. (B) Graphical representation of genotypes in polymorphic sites of *Gyrodactylus turnbulli* localized in scaffold scf7180000443747 (707 SNPs). Genotypes identified as reference homozygotes are shown in green, alternative homozygotes in red and heterozygous in yellow. Strains collected in 2017 and 2018 are shown in grey and black, respectively, and those collected from different local populations are separated by horizontal grey lines [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Spring Site). In contrast, in most Tobagonian strains, heterozygosity was elevated genome-wide (Figure 1D) and heterozygous genotypes were very similar for all samples (Figure 2), suggesting that these genomes represent ‘frozen hybrids’. Indeed, our analyses imply that no recombination occurred since the hybrids were formed. If recombination happened before expansion of the hybrid genotype, some regions with loss of heterozygosity would be found and shared for all HC and DR strains. We did not find any evidence for loss of heterozygosity in HC nor DR (Figure S8). In contrast to HC and DR, patterns suggesting recombination were obvious in some strains from the Spring Site population. Strains G328, G367 and G368 showed nonidentical patterns of heterozygosity across the nuclear

genome, consistent with recent independent outcrossing/selfing events followed by recombination. Two of these three Spring Site strains (G328 and G368) have a mitochondrial genome significantly divergent from other hybrids, suggesting that subsequent rounds of outcrossing occurred between hybrid and nonhybrid individuals, the latter carrying the divergent mtDNA. On Trinidad, a single individual showed slightly elevated heterozygosity, possibly as a result of sexual reproduction between two moderately diverged strains (G308, Figure 1D, Figure S6), and indeed, another individual from the same population showed a pattern consistent with being a subsequent generation recombinant (G312). Thus, in contrast to a set of pure hybrid populations we found on Tobago (Dog River and Health

Centre) recombination was not completely absent in other populations both on Tobago (Spring Site) and on Trinidad. This evidence is consistent with the data from laboratory cross infections with *G. turnbulli*, which demonstrated that in experimental infections with two strains, 4–11% of offspring were produced by outcrossing of the two strains (Schelkle et al., 2012).

Not only sexual reproduction but also most forms of parthenogenesis involve meiotic recombination, which is expected to lead to loss of heterozygosity spanning large genomic regions. However, automictic parthenogenesis (automixis) with central fusion can freeze heterozygosity in spite of recombination (Engelstädter, 2017; Jaron et al., 2018). Under automixis with central fusion, the probability of recombination increases with distance from the centromere, and particularly regions distal to the centromere are affected if recombination does occur. While we were not able to identify centromeres/telomeres in the reference genome of *G. turnbulli*, we did not find any evidence for large genomic regions with loss of heterozygosity (Figures S8 and S9). Thus, either Tobagonian hybrids (a) reproduce only mitotically, (b) recombination is strongly suppressed during automictic parthenogenesis, or (c) recombinants are removed by selection leading to clonal-like evolution. Our data suggest that the inferred, clonally-like reproducing hybrids spread relatively recently across Tobago, as indicated by high similarity of their mitochondrial genomes, corresponding to 2,700 (95% HPD: 1,500–4,000) years of divergence (Figure 3). The divergence time is based on the indirectly derived substitution rate in a single mitochondrial locus of *Gyrodactylus salaris* (13.1% per Mya; Kuusela et al., 2007). Other indirect estimates suggest lower (5.1% per Mya; Hahn et al., 2015) or higher substitution rates (up to 20.3% per Mya; Meinilä et al., 2004) in *Gyrodactylus* species, and therefore, divergence dating should be taken with caution. Nevertheless, low nucleotide divergence between mitochondrial genomes indicates a recent origin of the hybrids, and their widespread occurrence across Tobago provides evidence for their evolutionary success. Hybrid genomes that were most widespread on Tobago did not show any signs of recombination, and apparently remained stable, as would be expected under predominantly clonal evolution.

What evolutionary forces are responsible for the apparent success of clonal hybrids on Tobago? Tibayrenc and Ayala (2012, 2017) proposed that clonal evolution is a prominent feature of parasitic lineages and that selection has favoured reduced recombination as a strategy to minimize the disruption of favourable gene combinations. Their hypothesis considers the total set of reproductive strategies used by pathogens to escape 'recombination load'. In contrast, the 'epidemic clonality model' (Smith et al., 1993) advocates for the occurrence of occasional bouts of clonally reproduced strains in otherwise recombining species. Ephemeral clones are frequently replaced by other genotype combinations, maintaining a dominant role of sexual reproduction in evolution. Complex patterns of outcrossing events in *G. turnbulli* populations seem to be consistent with an 'epidemic clonality model' of evolution.

In the case of *G. turnbulli*, a bout of clonality is associated with hybridization between two, relatively divergent strains, confirming

that hybridization of parasites can lead to the emergence of highly successful, and potentially virulent strains. The potential to increase the fitness of *G. turnbulli* by combining different genomes was suggested in an earlier report of laboratory experiments, in which infection with two inbred strains led to higher parasite load compared with single-strain infections (Schelkle et al., 2012). However, the genomic divergence between these strains was unknown, and as discussed by the authors, this result could be due to competition between clones leading to the evolution of increased virulence (Schelkle et al., 2012).

Human activity, migrations and climate change shift the geographic distribution of many species (Brooks & Hoberg, 2007; Cable et al., 2017; Lafferty, 2009), which increases the probability of hybridization followed by adaptive evolution. This could be the case for *G. turnbulli*. Guppies are commonly kept in aquaria, and *G. turnbulli* are regularly reported in aquarium populations (Maceda-Veiga & Cable, 2019) from where they can potentially be released to nature. Alternatively, hybrids might have appeared naturally. While source populations of individuals that formed the hybrid are unknown, our approximate dating of divergence of mtDNA between Trinidad and Tobago is consistent with the end of the last glacial maximum, when a land bridge that probably existed between the islands during glaciation-related sea level decline was disrupted (Alexander et al., 2006; Lambeck, 2004). This land bridge, while present, might have facilitated migration of guppies between the islands and possibly other fish species capable of spreading *G. turnbulli* (see Cable et al., 2013; King & Cable, 2007).

Our study on the natural populations of another monogenean parasite infecting guppies (*Gyrodactylus bullatarudis*) suggested that recent hybridization has also played a predominant role in shaping genetic variation, but in that case in populations from Trinidad (Konczal et al., 2020). Hybridization between two diverged lineages and its subsequent recombination resulted in a mosaic genome composition of *G. bullatarudis*. Around half of the genome originates from one or another lineage. High divergence between recombined lineages and relatively little divergence between local populations led us to conclude that recent hybridization was followed by rapid expansion of a recombinant *G. bullatarudis* strain. Similar patterns of successful admixed hybrid genotypes have been reported for many other parasites, including schistosomes (Kincaid-Smith et al., 2019; Platt et al., 2019), *Trypanosoma* (see Tihon et al., 2017) and *Leishmania* (see Rogers et al., 2014) species, as well as Dutch elm disease pathogens (Brasier, 2001; Hesseuauer et al., 2020) and other fungal pathogens (Mixão & Gabaldón, 2018) highlighting the role of recombination between divergent genomes in parasite evolution. Our analysis of *G. turnbulli* genomes indicates that unlike its congener *G. bullatarudis*, natural expansion occurred without prior recombination. This scenario is similar to suggestions for *Gyrodactylus salaris*, a significant pathogen of Atlantic salmon. The Baltic salmon-specific *G. salaris* was proposed to have originated from a single hybridization event between two clades, followed by the clonal reproduction of 'frozen hybrids'; however, this suggestion was based on the analyses of a single anonymous nuclear marker



(Kuusela et al., 2007). Therefore, not only clonal expansion of hybrids, but also linkage to lethal variants for both alleles (balanced lethal system), or epistatic interactions could potentially explain the pattern observed in *G. salaris*. Our genome-wide data suggest that clonal expansion of 'frozen hybrids' can indeed take place among gyrodactylids.

Similar observations in other pathogens suggest that 'frozen hybrids' might have a significant evolutionary advantage. An interesting example comes from a common fungal parasite of amphibians, *Batrachochytrium dendrobatidis*, in which asexually reproducing hybrids have higher virulence than both parental strains in some host species (Farrer et al., 2011; Greenspan et al., 2018). That hybrid is highly heterozygous and widespread. Most likely contact between previously genetically isolated allopatric populations of the fungus facilitated hybridization, resulting in generation, spread and invasion of the hypervirulent strain (Farrer et al., 2011). However, the strain is not completely clonal and diversification can proceed by either mitotic or sexual recombination. Similarly, in *Toxoplasma gondii* and *Sarcocystis neurona* outcrossing can precede a disease outbreak (Grigg et al., 2001; Wendte et al., 2010), but subsequent selfing events were responsible for epidemic expansions, and as pointed out by the authors, it is not clear whether an out-cross is independently sufficient to cause an epidemic (Wendte et al., 2010). Our data demonstrate that a hybridization event has produced genotypes with an apparent advantage compared to nonhybrid strains, potentially via masking of recessive, deleterious mutations and/or allowing a widening of the ecological niche, and importantly, that the genomic diversity gained through this initial hybridization appears to have been largely conserved ('frozen') by suppression of and/or selection against recombination.

Parasites may be particularly prone to accumulation of deleterious mutations in their genomes (Criscione & Blouin, 2005). In general, the rate of accumulation depends primarily on the effective population size. In small populations, random genetic drift can overpower selection making it easier for deleterious mutations, particularly those with small effects, to become fixed (Kimura et al., 1963). This can cause mutational meltdown leading to population extinction (Lynch & Gabriel, 1990). Several features of macroparasite life cycles can act in concert to reduce effective population sizes and increase the risk of such meltdown (Criscione & Blouin, 2005). In particular, frequent bottlenecks associated with colonization/recolonization of new hosts can significantly reduce the efficiency of selection in removing mildly deleterious mutations from populations. Additionally, observations of *G. turnbulli* in the wild suggest that census population sizes are often small and fluctuating over time (Stephenson et al., 2015), which is consistent with the low heterozygosity and generally positive inbreeding coefficient in the nonhybrid populations that we investigated (Figure 1E, Figure S6). If this leads to homozygosity for a number of recessive or partially recessive mutations, hybridization between divergent strains carrying those mutations at different genomic locations would result in hybrid vigour. Alternatively, the

advantage of hybridization can be due to alleles carried by heterozygotes being adapted to different and a wider range of environments. In the case of parasites, divergent alleles may each allow infection of different host species or genomes, allowing heterozygotes to infect a wider range of hosts. For example, laboratory experiments on tapeworms in sticklebacks showed that two genetic lines were each only able to infect a single host species, whereas their hybrids could infect both species (Henrich et al., 2013). In the case of *G. turnbulli*, however, hybridization occurred among two strains infecting the same host species.

Clonal expansion of hybrids is likely facilitated by facultative sexual reproduction of *Gyrodactylus* species. In such systems, switching between facultative and obligate clonal reproduction does not appear to be evolutionarily constrained and can easily evolve when favoured by selection (Neiman et al., 2014). In the case of *G. turnbulli*, however, recombination is not completely suppressed, because it was observed in one of the hybrid populations on Tobago (Spring Site), where it might have occurred between hybrid and nonhybrid individuals. The fact that recombinant individuals are rare on Tobago might result either from rarity of recombination events, or from effective removal of recombinants from the population by selection. The disadvantage of recombinants may stem from restoring homozygotes for recessive deleterious mutations. Discrimination between these two possibilities ideally requires direct estimates of recombination rate, possibly based on changes in genotype frequencies over time in both hybrid and nonhybrid populations (Becheler et al., 2017).

Recombination can, at least to some degree, enable independent evolution of different genomic regions, allowing identification of genomic regions with elevated genomic variation between the two sampling years, possibly driven by selection. We therefore searched for such regions in Trinidad populations and found that one genomic region showed an extreme pattern of genetic diversity, with several interesting genes previously associated with modulating lifespan, stress resistance (methyltransferase NSUN5; Schosserer et al., 2015) and host-parasite interactions (Prohibitin; Jain et al., 2010) in other taxa. Future work including more individuals and experimental validation is needed to confirm involvement of these genes in co-evolutionary dynamics.

Concluding, our population genomic data set 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 whether 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 may be sufficient to cause pathogen expansion, thus emphasizing that such processes can result in the

emergence of invasive pathogens, some of which may be of public health and conservation concern.

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## 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; and J.R. and J.C. contributed to manuscript writing. All authors corrected the manuscript and approved the final version.

## DATA AVAILABILITY STATEMENT

The raw sequences are available as FASTQ files in the GenBank (BioProject Accession No. PRJNA603444).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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