INTRODUCTION

Killifishes are widely distributed across the globe and have successfully colonized a range of habitats, from coastal lagoons to rivers, streams, seasonal water ponds, as well as remote tropical islands, such as the Seychelles (Murphy & Collier, 1997). While annual killifish can colonize arid savanna environments by virtue of their desiccation-resistant embryos that can sustain life during months-long dry seasons (Cellerino et al., 2016; Dolfi et al., 2019; Hu et al., 2020), we know little about the biotic and abiotic factors that enabled killifish to colonize and adapt in islands that are hundreds of kilometres distant from the mainland, such as Madagascar and the Seychelles.

The mechanism proposed to explain the current distribution of killifishes across the planet is the Mesozoic divergence of the major
killfish clades, which followed the tectonic drift of Gondwana (Costa, 2013; Murphy & Collier, 1997). However, the genus *Pachypanchax* challenges this model. *Pachypanchax* are nonannual killfishes restricted to Madagascar and the neighbouring Seychelles, with the sister genus *Aplocheilus* found in India and Southeast Asia. Only a single species, *Pachypanchax playfairii* (the golden panchax) is present on the Seychelles islands.

Despite the current geographical proximity to Madagascar—which is geologically linked to continental Africa—the Seychelles islands have a closer geological relationship with the Indian subcontinent (Plummer & Belle, 1995; Seton et al., 2012). Hence, based on the continental drift hypothesis of taxon distributions, species from the Seychelles with limited dispersal capacity, like freshwater fish, are expected to have closer phylogenetic proximity to India than to Madagascar. However, the Seychelles and Madagascar are home to fish of the same genus (*Pachypanchax*) based on morphological traits, suggesting the possibility for post-tectonic transoceanic dispersal as a mechanism for species dispersal. If taxa mainly speciated as a consequence of geographical isolation due to tectonic events, the phylogenetic branching order and timings are expected to match those of continental drift.

In this study, we investigate the mechanisms of species dispersal in killfish—with a focus on the golden panchax from the Seychelles archipelago—and we explore how long-term isolation and recent population size decline have shaped their genome.

Phylogenetic and population genomic methods can help directly test whether tectonic or transoceanic dispersal can explain the evolution of the golden panchax. Moreover, adaptations enabling salt tolerance may have led to transoceanic migrations, which is an experimentally testable hypothesis tackled in our study.

After exploring the causes of island isolation in fish of the genus *Pachypanchax*, we explored its consequences. The multimillion-year evolutionary history of isolation on the Seychelles archipelago for golden panchax, combined with its presumed limited dispersal capacity, represents a paradigmatic example of island evolution for a vertebrate (MacArthur & Wilson, 2001). Island isolation leads to inbreeding, which makes species susceptible to extinction (Frankham, 1998). Reduction of effective population normally lowers the efficacy of selection, leading to the accumulation of deleterious mutations (Charlesworth et al., 1993). Moreover, shifts in the ecological niche can also lead to relaxed selection by removing niche-specific selective constraints (Wertheim et al., 2014). However, little is known about the genomic consequences of long-term island isolation.

Previous studies conducted in continental African killfishes have shown that relaxation of selection dominates genome evolution in short-lived annual species, preferentially targeting ageing-related pathways, probably resulting from population fragmentation and population size reduction, combined with a strong selection for early-life phenotypes, such as embryonic diapause and explosive sexual maturation (Cui et al., 2019; Willemsen et al., 2019). Here, we investigate the impact of island isolation on genome evolution and relaxation of selective constraints in a long-lived killfish.

## METHODS

### 2.1 Sample collection

We added new data for *Pachypanchax omanolotus*, *Nothobranchius virgatus* and a population of *Pachypanchax playfairii* to our previous data set (Cui et al., 2019). Specimens from most populations were collected during a dedicated field expedition in April 2016 (and by E.H. during the following month), using dip nets. Fish were identified in the field, and small fin clips were taken from their caudal fin and stored in 98% ethanol. Fish were then released back to their habitat. Voucher specimens (a random subsample of both sexes) were taken from most populations. All field sampling and export procedures followed regulations of the Seychelles, with permits and research associateship issued by Seychelles Bureau of Standards Ref. A0157 dated March 22, 2016. Samples from Mahé North were from aquarium strains bred in captivity since 2013, for approximately three to four generations (Table S1). The outgroup species *Pachipanchax omanolotus* is a captive-bred individual derived from Sambirano-Ramena, Madagascar.

### 2.2 Library preparation and sequencing

Library preparation follows previous methods (Cui et al., 2019; Rowan et al., 2015). Protocol details are given in the Supporting Information.

### 2.3 Read processing and genotype calling

Paired-end reads were adapter- and quality-trimmed with TRIMMOMATIC version 0.32 (Bolger et al., 2014) before being processed to generate variant calls using a combination of PICARD tools, GATK and BCFTOOLS (Appendix S1).

### 2.4 Genome size estimation of *P. omanolotus*

As previously described, we mapped the trimmed reads of *P. omanolotus* to a draft genome of *P. playfairii* with BWA-MEM, counting read coverage in the single-copy Benchmarking Universal Single-Copy Orthologues (BUSCOs). Genome size was calculated as total read base pairs divided by read coverage. This simple method has been shown to agree well with estimates of genome size by flow cytometry and to be reliable independently from the choice of the reference genome or to read coverage (Cui et al., 2019).

### 2.5 Phylogeny and molecular dating

To infer the divergence dates of key nodes in the killfish phylogeny, we adopted a relaxed molecular clock method calibrated by externally inferred teleost node dates (Near et al., 2012). A detailed description is given in the Appendix S1.
2.6 | Population genetic statistics

To gain an overview of the population genetic parameters of different *P. playfairii* populations, we computed $F_{ST}$, $d_s$, and $\theta_s$ statistics. All genomic regions, including protein-coding and noncoding, are included. The gVCF files of individuals were merged with the merge command in bcfTools version 1.6, keeping missing genotypes as missing. The merged gVCF file was converted to geno format with the parseVCF.py script from the genomics_general package, requiring a minimal variant quality of 60. A read coverage filter was applied to include only regions within [0.025, 0.975] quantiles. We used the popgenWindows.py script (https://github.com/simonhmartin/genomics_general) to compute $F_{ST}$, $d_s$, and $\theta_s$ for all *P. playfairii* populations in 20-kb nonoverlapping windows, with the requirement of at least 5000 bp of nonmissing data. Mean statistics across all windows are reported.

2.7 | TREEMIX analysis

To infer the order of population separation and potential gene flow from allele frequency data between *P. playfairii* populations, we adopt the TREEMIX method (Pickrell & Pritchard, 2012). We included *P. playfairii* samples (Table S1) from seven localities on the Seychelles: Pasquiere River (Praslin, population A, n = 2), Plaine Hollandaise (Praslin, population B, n = 1), Praslin West (population C, n = 4), La Digue (population D, n = 2, captive sample), Curieuse (population F, n = 3), Mahé North (population E, n = 5, captive samples) and Mahé South (population G, n = 3). All genomic regions, including protein-coding and noncoding, are included. We used a single captive individual of the outgroup species *P. omalonotus* derived from the northwest part of Madagascar. Distributions of read coverage at each population level were estimated from merged BCF files. BCF files were then converted into the TREEMIX (Pickrell & Pritchard, 2012) allele count format with a custom script, excluding sites with read coverage below the 0.025 or above the 0.975 coverage quantiles. The OPTM R package (Fitak, 2019) was used to determine the optimal number of migration edges. TREEMIX was run with 0–6 migration edges in 20 iterations, starting from k = 200 to 4000, with an increment of 200 per iteration. The output files from TREEMIX were used as input for OPTM, where the Evanno method was used to estimate the proportion of variance explained by different number of migration edges. The ad hoc statistics $\Delta m$ was used to select for the optimal number of migration edges. We ran this analysis either with or leaving out the outgroup species *P. omalonotus*.

2.8 | Estimating population separation times with G-PHOCS

TREEMIX results inform the order of population splits and direction of gene flow, but lack direct information on split times. To jointly infer divergence times, ancestral population sizes and level of gene flow between *P. playfairii* populations, we used the G-PHOCS method (Gronau et al., 2011), which uses a Markov chain Monte Carlo (MCMC) procedure to infer model parameters from unlinked neutral alignments. The following formulae were used to convert the raw output to population genetic parameters. Using $\mu = 3.72 \times 10^{-9}$ and 1 generation/year, we have $\tau = \text{Output}_\tau/\text{tau-theta-print/mu}$; generation time, $\theta = \text{Output}_\theta/\text{tau-theta-print}$ and $\text{prob(hybrid origin)} = 1 - \exp\left(-\mu \times \text{Output}_m/\text{mig_rate_printfac}\right) \times (\text{tauABC} - \text{tauBC})$. Further details are given in the Appendix S1.

2.9 | PSMC analysis

To investigate the change of effective population size in *P. playfairii* populations in the recent timescale of ~10,000–500,000 years, we applied the PSMC’ method on individual diploid *P. playfairii* genomes, following the msmc2 (Schiffels & Durbin, 2014) manual. All genomic regions, including protein-coding and noncoding, are included. Briefly, sequencing depth was estimated by averaging the per-base coverage computed by the SAMTOOLS depth command with no mapping quality cutoffs. The bamCaller.py tool from the msqc-tool package was used to call variants and generate a mask of regions with abnormal coverage. The files were then converted to msmc2 format by the generate_multihetsep.py tool. The PSMC’ method was run for each individual’s pseudochromosome separately using unphased genotypes in msmc2, with an initial rho/mu set to 2.61976354 based on mu estimated from the dated phylogeny and the assumption of two recombinations/meiosis/chromosome, as informed by previous genetic maps in cyprinodontiforms (Schartl et al., 2013; Valenzano et al., 2015). The initial mutation rate and recombination rate were then jointly inferred by the program during the run from data.

2.10 | Asymptotic McDonald–Kreitman analysis and direction of selection

To examine whether recently declining population size resulted in accumulation of deleterious mutations of the protein-coding genome in *P. playfairii*, we collected individual golden panchax from several natural localities (Table S1), sequenced their genomes and conducted analysis based on the McDonald-Kreitman test, using *P. omalonotus* as an outgroup. We pooled individual fish sequences from Praslin, Curieuse and La Digue to analyse the asymptotic McDonald–Kreitman alpha (Messer & Petrov, 2013). Calculations of the MK and DoS (direction of selection) statistics were as previously described (Cui et al., 2019), where MK alpha = 1 – (pN/pS)/(dN/dS) and DoS = dN/(dN + dS) − pN/(pN + pS). A positive alpha value is an estimate of the proportion of substitutions fixed by positive selection since divergence from *P. omalonotus*. A negative alpha/DoS is due to the segregation of slightly deleterious mutations in *P. playfairii* since divergence from the outgroup (Messer & Petrov, 2012).
2.11 | Neutral simulations with scrm

To validate the robustness of treemix and MK tests in our sampling scheme, we performed scrm (sequential coalescent with recombination model) simulations (Staab et al., 2015) in a strictly bifurcating tree using tau and theta parameters inferred by g-phocs as inputs (Figure 1b) and simulated the same number of individuals per population as the real data. A detailed description of the methods and results is given in the Supporting simulations.

2.12 | RELAX tests

To examine the intensity of natural selection of protein-coding genes at a macro-evolutionary timescale, we turned to the relax method (Wertheim et al., 2014). Only single individuals were kept for relax (Wertheim et al., 2014) and busted-mh (Wisotsky et al., 2020) tests. We added the newly sequenced P. amalonotus and N. virgatus into our previous orthologue data set (Cui et al., 2019). We developed a reparameterized two-scaler relax method, and validated its performance using simulations. Further details are given in the Supporting simulations.

2.13 | Positive selection test with BUSTED-MH

To investigate whether the proportion of protein-coding genes under intensified selection at a macro-evolutionary timescale can be attributed to positive selection, we use an improved version of the BUSTED (Murrell et al., 2015) method implemented in hyphy. Detecting positive selection in protein-coding sequences has recently been shown to be vulnerable to two violations in model assumptions, namely instantaneous multinucleotide substitutions (Venkat et al., 2018) and site-to-site variation in synonymous substitution rates (Wisotsky et al., 2020). We thus use a developmental version of the BUSTED test that accounts for both processes, named BUSTED-MH, in order to test for positive selection in the genus Pachypanchax. This script implementing the analysis can be run with hyphy version 2.5.10 or later and is available from https://github.com/veg/hyphy-analyses/.
2.14 | Conserved element detection and PHASTCON analysis

While the above methods focus on the protein-coding part of the genome, we explored whether relaxed selection also occurred in noncoding conserved proportions of the genome. We performed a whole-genome alignment using PROGRESSIVE CACTUS (Paten et al., 2011) of four species in three annual genera (Austrofundulus, Callopanchax and Notobranchius), and one nonannual species (P. playfairii). PhastCons in the R PHAST package (Hubisz et al., 2011) was used to identify conserved genomic regions in the annual species, omitting the nonannual. Due to the long divergence of these annual genera, the conserved genomic regions probably contain functionally important regulatory or protein-coding elements. After excluding protein-coding elements, we then identified accelerated noncoding regions in P. playfairii that are otherwise conserved in annuals by comparing the rate with four-fold degenerate sites with PhyloFit in the PHAST package. Because four-fold degenerate sites evolve slower than neutrality in many organisms (Künstner et al., 2011; Lawrie et al., 2013), accelerated conserved elements (ACEs) detected in this manner probably reflect relaxed selection.

2.15 | Estimating synonymous substitution rates using CODEML

To examine whether ACEs are located in regions with low recombination, we take advantage of the fact that mutation rate in a wide range of organisms (e.g., Halldorsson et al., 2019), including African killifishes (Cui et al., 2019), is positively correlated with local recombination rate. We estimated dS by extracting the P. omalonotus and P. playfairii alignments from the BUSTED-MH data set, and running them through the pairwise model in CODEML. The obtained dS values were then used to correlate with upstream ACEs at different distances, compared to randomly sampled genomic regions.

2.16 | DREME analysis

To test whether the accelerated conserved regions in Pachypanchax contained functional sequences in their ancestral states, we extracted the 50-bp Pachypanchax-ACE sequences from the homologous locations in N. furzeri, and used DREME (Bailey, 2011) to analyse the enrichment of hexamers. A literature search was performed to check whether any of the enriched hexamers corresponds to known functional regulatory motifs.

2.17 | Gene ontology and pathway analysis

Gene ontology (GO) and pathway enrichment analyses were performed on the ConsensusPathDB website (Herwig et al., 2016). Genes called as relaxed ($p < .05$) were mapped to Ensembl IDs of the human orthologue, and compared to a background list of all genes that were entered into the RELAX analysis. For the ACEs, the immediately downstream protein-coding gene was used as the foreground, and a random set of genes sampled from the genome which are more than 170 kb away from any ACEs was used as the background.

2.18 | Incubation of killifish embryos in seawater

To test whether freshwater P. playfairii tolerate seawater and could in principle cross salt water barriers, we directly compared the viability of killifish embryos incubated in autoclaved fish room water and artificial seawater (33 g RedSea Marine Salt in 1 L RO water). Four species/strains were examined: P. playfairii, N. furzeri GRZ (two strains, GRZ-AD and GRZ-Bell, independently derived from Gonarezhou National Park, Zimbabwe) and MZCS-002 (Bartakova et al., 2015). For each species or strain, fertilized eggs produced within 1 week were collected from three of four breeding tanks each consisting of one male and three or four females. Eggs in batch 1 were bleached with 0.5% hydrogen peroxide to prevent fungal infections. We used $n = 100$ eggs per $N. furzeri$ strain and $n = 117$ eggs for $P. playfairii$, with $-25-30$ eggs per Petri dish. Batch 2 was washed in autoclaved freshwater but not bleached. Batch 2 contained 320 GRZ-Bell eggs, 140 MZCS-002 eggs and 94 $P. playfairii$ eggs, incubated at a density of 23–40 eggs per Petri dish. In both batches, embryos were checked daily. Dead embryos were recorded and removed and medium was exchanged. Hatched $P. playfairii$ were transferred to the hatching incubator and slowly acclimatized to freshwater conditions if they were hatched in seawater (by replacing 50% of the artificial seawater with freshwater daily). $N. furzeri$ embryos were transferred to damp Whatman paper after reaching the black eye stage for incubation. All embryos were incubated at 28°C. The logrank test was used to test for survival differences among the tested groups.

2.19 | Pachypanchax post-embryonic development in seawater

Two small (9-L) standalone tanks, one containing freshwater from the main fish system and one containing seawater (33 g sea salt L$^{-1}$ RO water) were set up with internal air-driven box filters. Temperature was maintained at 26°C. Two batches of 12 fertilized eggs each from a freshwater breeding tank were placed into each of the experimental tanks. Hatched fish were fed brine shrimp. After 2 months the experiment was concluded as males in either tank displayed sex-specific colour changes.

3 | RESULTS

3.1 | The killifish biogeographical pattern supports transoceanic species dispersal

We constructed a phylogenetic tree using 4026 orthologous protein-coding sequences from 61 teleosts, adding the newly sequenced genome of the Madagascar species Pachypanchax omalonotus
(estimated genome size ~707 Mbp). Based on calibrations obtained from a broader-scale teleost phylogeny (Near et al., 2012), we inferred divergence times with mcmctree (Figure 1a). South American representatives of the family Rivulidae diverged from African and Indo-malagasy families ~59.85 million years ago (Ma) (95% confidence interval [CI] 52.66–67.07). Nothobranchiidae, which are found in continental Africa, diverged from the Indo-malagasy Aplocheilidae ~52.90 Ma (95% CI 46.33–59.66). Continental African killifishes are deeply diverged into western and eastern clades ~38.64 Ma (95% CI 31.55–43.50). Amongst all sampled genera with more than one representative species, Pachypanchax has the most ancient common ancestor at ~14.95 Ma (95% CI 11.47–18.77), even deeper than the divergence between the sister genera Nothobranchius and Pronothobranchius, which dates to ~8 Ma (95% CI 6.87–9.22), and between Callopanchax and Scriptaphyosemion, which dates to ~8.9 Ma (95% CI 7.61–10.32) (Figure 1a). The divergence between the two extant species in the genus Pachypanchax is comparable to the divergence between more distantly related genera, such as Fundulopanchax and Nothobranchius (14.4 Ma, 95% CI 12.59–16.50) or Archiaphyosemion and Callopanchax (14.5 Ma, 95% CI 12.45–16.41). To our surprise, neither the order of divergence nor any of the major divergence dates fit the known tectonic order of the Gondwanan continents, suggesting that transoceanic dispersal rather than tectonic drift may explain the current biogeographical pattern within killifish (Figure 1a). We also found that although the concatenated mitochondrial tree using codon positions 1 and 2 has only a moderate rapid bootstrap support of 69 and a nonsignificant AU test statistics of 0.264 (Shimodaira, 2002), the topology of the maximum-likelihood tree agrees with the nuclear genome (Figure S1), a result contradicting previous mitochondrial trees built with fewer genes (Murphy & Collier, 1997). Therefore, we find that the tree topologies from mitochondrial and nuclear genetic data are largely in agreement across all the major branches of killifish and support a significant contribution of transoceanic species dispersal to the current killifish species distribution.

Studying population separation among golden panchax (Pachypanchax playfairii) within the Seychelles reveals a branching pattern consistent with island distance (TREEMix analysis, Figure 1b); and the genome-wide statistics of divergence $\theta_y$ and $F_y$ agree with the migration analysis based on TREEMix tree (Figure 1b). Overall, the level of divergence between populations has a similar range as within-population polymorphisms ($d_y$, ~0.0012–0.0034, $\theta_y$, ~0.001–0.0023), suggesting recent divergence. G+PHOCs inference of divergence times confirms a very recent separation between sampled P. playfairii populations (Figure 1b). The split between P. playfairii populations on Mahé and other islands is dated to only ~90.3 thousand generations ago, orders of magnitude later than the separation of the Seychelles islands (Plummer & Belle, 1995). The separation between La Digue and Praslin+Curieuse populations was merely ~35.25 thousand generations ago, shortly pre-dating the subsequent separation between Praslin and Curieuse populations at ~35.10 thousand generations ago. Interestingly, samples from northern Mahé island are relatively divergent from samples from the south at around 84.3 thousand generations ago. TREEMix analysis supports adding one migration edge from the La Digue population to the (B+C) population on the neighbouring Praslin island, with an estimated edge weight of 39.3–41%, suggesting high gene flow (Figure 1b). The gene flow from D to the ancestor of B/C estimated by G+PHOCs is similar, at about 36%. The resulting divergence among populations remains unchanged with outgroup inclusion (Figure S2). We note that samples of B and C are directly wild-caught, precluding laboratory contamination. Addition of further migration edges is not supported as even when one migration was added, the network already explains on average 99.5% (outgroup excluded) or 99.99% (outgroup included) of the variation in the data (Figure S2). SCRB simulations assuming a strictly bifurcating population history using G+PHOCs-estimated parameters, or additionally with highly reduced population sizes in recent generations for D and E to mimic domestication, do not result in false inference of gene flow in TREEMix + OPTM (Figure S2E), suggesting that the gene flow signal detected in the real data cannot be simply explained by our sampling scheme.

Together, our results suggest that both macro- and micro-evolutionary divergence of killifishes can be in part explained by transoceanic dispersal.

3.2 | Pachypanchax are able to develop in seawater

To assess whether golden panchax would survive extended periods of time in the sea, which would be consistent with the transoceanic species dispersal supported by the genomic data, we incubated in seawater golden panchax embryos and compared their developmental trajectory to embryos raised in normal conditions (i.e., freshwater incubation). To our surprise, we found that golden panchax embryos develop perfectly in seawater, with no detectable differences in survival with control groups incubated in freshwater (Logrank test, $p_{\text{no-bleach}} = .65$, $p_{\text{bleached}} = .4$) (Figure S3). Moreover, fry from golden panchax embryos raised in seawater remain viable. This finding does not extend to the continental African killifish Nothobranchius furzeri, whose embryos did not survive in seawater, with all embryos failing to survive 3 days after collection (Logrank test, $p < 10^{-6}$). In a follow-up experiment, P. playfairii fry successfully develop up to sexual maturation in artificial seawater after 2 months before being transferred back to freshwater (100% survival for the hatched fry). The same applies to the freshwater controls, which reached sexual maturity in the same time frame. Our findings suggest that golden panchax could in principle sustain extended periods of time in seawater, which is compatible with a model of direct transoceanic dispersal via ocean currents.

3.3 | Annual killifishes from Madagascar and Seychelles accumulate deleterious mutations as population size declines

Annual killifishes in the genera Nothobranchius and Callopanchax from continental Africa display a larger proportion of genes under relaxed selection.
rather than intensified selection compared to their most closely related nonannual species in Aphyosemion and Scriptaphyosemion (Cui et al., 2019). We investigated whether the nonannual genus from Madagascar and Seychelles (i.e., \textit{P. omalonotus} and \textit{P. playfairii}) has a similar proportion of genes under relaxed versus intensified selection observed in the annual or nonannual killifish species from continental Africa. We first used phylogenetic methods to examine the intensity of selection at the macroevolutionary scale. We identified 444 genes under relaxed purifying selection ($p < .05$, Table S4) and 877 genes under intensified selection ($p < .05$, Table S5) in the genus \textit{Pachypanchax} relative to other aplocheiloids. Compared to genera of annual killifish within the family Nothobranchiidae, the genus \textit{Pachypanchax} has a larger proportion of intensified rather than relaxed genes (Figures 2a and 3) than two annual genera \textit{Nothobranchius} and \textit{Callopanchax}, but at a similar level as the nonannual killifish genus \textit{Scriptaphyosemion} (from continental Africa, sister to the annual genus \textit{Callopanchax}). Overall, our findings show that the island annual species of the genus \textit{Pachypanchax} have a larger proportion of genes under intensified rather than relaxed selection compared to annual killifishes, to a level that is comparable to nonannual killifish genera living in continental Africa. Nevertheless, the total number of genes (444) under relaxed selection in \textit{Pachypanchax} is still much higher than the simulated false positive rate (Table S3; expected = 253–269 in 10,556, assuming 1%–10% intensified genes,

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**FIGURE 2** (a) Ratios plotted on a logarithmic scale of gene counts that are detected as relaxed and intensified selection in five killifish genera. Relaxed selection was detected by the 2-k parameterization, intensified selection by the original 1-k parameterization. Confidence interval obtained by 1000 multinomial resampling. Red line indicates ratio at 1. (b) McDonald–Kreitman $\alpha$ in \textit{Pachypanchax playfairii} plotted against derived allele frequencies (outgroup \textit{Pachypanchax omalonotus}). (c) psmc plot for wild-caught \textit{Pachypanchax playfairii} individuals from three localities in Praslin, one from Curieuse and one from Mahé.
90%–99% genes with no shifts in selection and 2.5% positively selected genes) of the test, suggesting that relaxed selection led to fixation of a small number deleterious mutations in this nonannual genus.

To investigate possible relaxation of selection in Pachypanchax over a more recent time frame, we focused on the more isolated Seychelles species golden panchax (P. playfairii). The estimated ratio of mutations fixed by positive selection estimated by the MK alpha is not significantly different from zero, suggesting not many sites were positively selected in this Seychelles taxon. At lower derived allele frequencies, the alpha statistics remains negative and does not reach the asymptotic level until 60% of derived allele frequency, indicating that slightly deleterious variants are segregating at low to intermediate frequency in the population (Figure 2b). Neutral simulations with SCRM mimicking our sampling scheme and population parameters confirm the robustness of the MK alpha statistics to population structure and unbalanced sample size from subpopulations; that is, no down-bias of the MK alpha statistics is observed at low to intermediate allele frequencies in simulations (Figure S2G–I).

Therefore, although the macroevolutionary trend suggests that the Pachypanchax clade has more intensified selection compared to annual killifishes from continental Africa, the MK alpha measurements indicate the presence of genetic variants at low to intermediate derived frequencies (i.e., not fixed), which are probably slightly deleterious gene variants accumulated over a more recent timescale.

We then investigated what could be the leading cause for the deleterious gene variants segregating at low to intermediate frequency in the golden panchax. A probable scenario is that decreased effective population size may have occurred in this island species. To test this hypothesis, we analysed individual genomes from wild-caught golden panchax using PSMC', and found that most populations underwent a gradual decline in effective population size after 200,000 generations ago, ending in a population size of about 5000–20,000 at generation 5000 (Figure 2c). Different individuals from the same population show repeatable PSMC’ traces. Population A from Praslin island had a population expansion from ~20,000 generations ago until ~7000 generation ago, after which it rapidly declined to less than 10,000. Overall, island annual killifish display a larger proportion of the genome under intensified rather than relaxed selection, compared to annual killifishes from continental Africa. However, their recent progressive reduction in population size may have caused the genome-wide accumulation of nearly neutral polymorphisms at otherwise conserved sites in more recent generations.

3.4 Relaxation of selection in noncoding regions

Besides protein-coding genes, the molecular evolution of regulatory elements of genomes underlies important phenotypic variations in response to selection in different organisms, including the loss of flight in ratite birds (Sackton et al., 2019) and degeneration of armour plates in stickleback fish (O’Brown et al., 2015). We therefore explored whether noncoding regions in the Pachypanchax genome experienced relaxation of selection. First, we identified 50-bp
conserved noncoding elements (totalling 86,087,429 bp, excluding CDS 56,842,251 bp, excluding repeats 54,779,098 bp) in the assembled genomes of the annual species *Austrofundulus limnaeus, Callopanchax toddi, Notobranchius furzeri* and *Notobranchius orthomolotus* using *phastcon*. These elements are expected to have highly conserved regulatory functions within an ~60 million year divergence time. Together, 10,223 (511.15 kb, or 0.093%) of these elements were significantly accelerated (i.e., had significant sequence divergence compared to the annual species) in the *Pachypanchax* genome when compared to four-fold degenerate sites. Because four-fold degenerate sites are known to evolve more slowly than completely neutral sites due to codon bias (Hershberg & Petrov, 2008), we interpret the majority of these accelerated elements to have probably undergone relaxed purifying selection in the genus *Pachypanchax*. We then investigated whether these accelerated conserved elements were enriched for regulatory sequence motifs (using *dreme*), and found enrichment for the promoter TATA box ([TAATTA, \( p = 5.6e-28 \)) and the enhancer box ([CAGCTG, \( p = 6.5e-13 \)), corroborating their potential role as regulators of gene expression. Then, we investigated whether these noncoding accelerated conserved elements were associated with downstream genes that evolved under intensified or relaxed selection. We found that protein-coding genes within 80 kb from an accelerated conserved noncoding element were more likely to be also under relaxed selection, according to the two-scaler *relax* test. Furthermore, the median direction of selection (*DoS*, where negative values are indicative of genes evolving under relaxed selection) was also more negative for protein-coding genes near an accelerated conserved noncoding element (Figure S4). Using a protein-coding gene synonymous substitution rate as an approximation of the local mutation rate, we found that the noncoding accelerated elements are located near or within genomic regions with a lower mutation rate (Figure S4D). In a wide range of taxa, including African killifish (Cui et al., 2019), mutation rate is positively correlated with recombination rate (Halldorsson et al., 2019). The finding is consistent with purifying selection being less effective in purging deleterious variants in low-recombinant regions of the genome due to Hill–Robertson interference. Overall, we found that relaxation of selection in *Pachypanchax* extends beyond the coding regions, supporting a scenario where gene regulation and adjacent coding sequences are both subject to fluctuations of evolutionary constraints.

### 3.5 | Intensified selection on RNA polymerase complex genes

We performed a GO term enrichment analysis on intensified genes (i.e., under positive or purifying selection) detected with the original *relax* method (more powerful in identifying genes under intensified selection, based on our simulations), after excluding genes detected to be under relaxed selection by the new parameterization (more powerful in identifying genes under relaxed selection, based on our simulations). Eight biological complexes were found to be enriched at a false discovery rate (FDR) cutoff of 0.05. The most significantly enriched component was the RNA polymerase complex \(( p = 2.12e-6, \text{ FDR} = 0.000338)\), containing 19 genes detected to have undergone intensified selection in the *Pachypanchax* branch, including six RNA polymerase subunits and four general transcription factor subunits. Other complexes under intensified selection (FDR < 0.05) include the MICOS complex, smooth endoplasmic reticulum, nuclear envelope lumen and transferase complex (Table S6).

### 3.6 | Relaxed selection affects genes involved in early *Pachypanchax* development

Despite having some portion of the genome under relaxed purifying selection, fish of the genus *Pachypanchax* live for several years, significantly longer than the corresponding annual species from continental Africa, whose lifespan can be as short as 4–6 months (Valenzano et al., 2015; Willemsen et al., 2020). Since relaxation of purifying selection largely affects genes involved in late-life maintenance in annual African killifishes (Cui et al., 2019), we investigated what genes and gene functions were affected by relaxed selection in a clade with long-term island isolation yet maintaining a normal lifespan. To answer this, we performed GO analysis on the genes under relaxed selection (Figure S5; Table S7). Terms related to early development, such as anatomical structure morphogenesis \(( p = 7.00e-5, \text{ FDR} = 0.0012)\), cell communication \(( p = 1.47e-6, \text{ FDR} = 5.19e-5)\) and cell surface receptor signalling pathway \(( p = 1.68e-7, \text{ FDR} = 6.51e-5)\), are strongly enriched in genes under relaxed selection. Further pathway analysis using *consensuspathdb* (Herwig et al., 2016) of the same gene set reveals that seven pathways are enriched (Table S8; FDR < 0.05), including immune system, adhesions junctions interactions and apoptotic cleavage of cell adhesion proteins. Among these is the fibroblast receptor 2 ligand-binding pathway \(( p = .000495, \text{ FDR} = 0.046)\), which has been found to be important for early embryogenesis. Aligning the protein sequences of human and killifishes, we found that both *P. omalotus* and *P. playfairii* carried a serine instead of a glycine at a critical position on *fgf10*, while this position is conserved from human to other fishes (Figure 4). This amino acid is homologous to G160 in human *fgf10*, which in turn interacts with R251 on the human receptor *fgfr2* (Yeh et al., 2003). The homologous amino acid R251 of *fgfr2* is conserved in all killifishes, including *Pachypanchax*.

As we found that relaxation of selection applies to both coding and noncoding regions, we investigated what genes and gene functions were enriched in proximity of accelerated noncoding elements in the genus *Pachypanchax*. This analysis confirmed that accelerated noncoding elements map in proximity of genes involved in developmental processes (Table S9). Therefore, not only did we find a strong correlation between relaxation of selection in coding and adjacent noncoding regions (Figure S4), but both GO enrichments support that nonannual fish of the genus *Pachypanchax* underwent relaxation of selection at specific developmental genes.
corresponding to human FGFR2 is conserved residue 160 in human FGF10. The key amino acid at position 251 have a unique substitution from glycine to serine corresponding to killifish species distribution through tectonic drift or transoceanic events is debated (Briggs, 2004). The Malagasy chameleon Archaius tigris was shown to be sister to a continental African chameleon genus, which did not diverge until the Eocene–Oligocene, again supporting the possibility for transoceanic dispersal (Townsend et al., 2011). Molecular dating of the now extinct Malagasy elephant bird reveals its relatively recent divergence with the sister group New Zealand kiwis, suggesting transoceanic dispersal by their flying ancestors (Grealy et al., 2017).

Killifish distribution has been suggested as an example of speciation following the sequence of events that led to the separation of the Gondwanan plates, largely supported by the congruence between mitochondrial phylogeny and the order of break-up of the geological tectonic plates (Murphy & Collier, 1997). However, a small-scale phylogenetic tree built on nuclear genomic information (Pohl et al., 2015) has presented a different branching pattern, placing continental African killifishes as sister species to the Indo-Malagasy clade, raising the question of whether tectonic drift is indeed the unique force explaining killifish distribution. Our whole-genome phylogenetic tree, based on the nuclear genome, rejects the proposal of a sister relationship between South American and continental African taxa, supporting that Indo-Malagasy and continental African taxa form a sister relationship. Furthermore, contrary to the species distribution predicted by the order of events congruent with tectonic drift, we found that Seychelles Pachypanchax are closely related to Malagasy taxa, rather than to the Indian genus Aplocheilus. Our molecular dating further shows that the 95% posterior distributions of divergence times are more recent than the proposed separation time of the corresponding continental plates. We also found some evidence of cross-island gene flow from La Digue to Praslin in P. playfaii, although the inferred timing of 25,000 years may be recent enough to overlap with potential early human activities, which we cannot exclude. Overall, a more likely explanation for the current distribution of killifishes probably involves oceanic barrier crossings (Figure S6). However, the mechanisms by which transoceanic migration might have occurred is unknown. Open-ocean “rafts” and dispersal by birds have been suggested as plausible means (Silva et al., 2019). In our work, we show for the first time that the freshwater golden panchax (Pachypanchax playfaii), a nonannual killifish from the Seychelles islands, can complete embryonic development, successfully hatch and develop to adulthood in seawater. Of note, we also found that killifish species from mainland Africa (genus Nothobrachius) cannot survive in seawater, suggesting that Pachypanchax may have evolved a specific adaptation that could have provided them with a means of direct transoceanic dispersal. Alternatively, saltwater tolerance could be an ancestral trait of all cyprinodontiform killifishes, but was secondarily lost in the continental

3.7 Minimal evidence of positive selection in Pachypanchax

To detect positive selection in the genus Pachypanchax, we ran BUSTED-MH (Table S9). Correcting for multinucleotide substitution and mutation rate variation, we found little evidence of positive selection in this clade, as less than 5% of tested genes were called as under positive selection, not exceeding the p value cutoff of 0.05 (Figure 3). GO analysis of candidate positively selected genes revealed no enriched terms below an FDR cutoff of 0.05 (Table S10). Therefore, the higher proportion of genes under intensified rather than relaxed selection in Pachypanchax is probably due to genes under purifying selection, rather than under positive selection.

4 DISCUSSION

4.1 Transoceanic dispersal is compatible with killifish species distribution

How do organisms with limited dispersal capacity, such as freshwater fishes, colonize distant islands? Whether this is achieved through tectonic drift or transoceanic events is debated (Briggs, 2003; Sparks & Smith, 2005). Indeed, evidence for transoceanic dispersal has been found in several groups of animals. For example, palaeontological and molecular clock analyses place the divergence time of cichlid fish in the Palaeocene (65–57 Ma), much later than Mesozoic tectonic rifting of Gondwana (Friedman et al., 2013). Based on molecular clock and nucleotide substitution saturation analysis in mitochondrial genes, Malagasy amphibian, reptilian and nonflying mammals are too closely related to continental sister groups to fit the ancient tectonic events (Vences, 2004). The Malagasy chameleon Archaius tigris was shown to be sister to a continental African chameleon genus, which did not diverge until the Eocene–Oligocene, again supporting the possibility for transoceanic dispersal (Townsend et al., 2011). Molecular dating of the now extinct Malagasy elephant bird reveals its relatively recent divergence with the sister group New Zealand kiwis, suggesting transoceanic dispersal by their flying ancestors (Grealy et al., 2017).

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African taxa via relaxed selection. Interestingly, the latter scenario would be compatible with transoceanic dispersal events during early killifish evolution, such as the crossing event predicted between South America and Africa (Figure 1). A broad salt-tolerance survey of killifish species would be required to identify the most likely evolutionary order of the trait.

4.2 | Recent decline of population size accompanied by limited relaxed selection and positive selection

Colonizing new island habitats is probably accompanied by both a reduction of effective population size and by a change of the ecological niche, which may affect a suite of phenotypes. Long-term evolution in islands, with consequent chances for population size reduction, could lead to relaxation of selection constraints. For instance, a sudden lack of predators in novel island environments can explain the loss of antipredator behaviours in oceanic tortoises (Austin & Nicholas Arnold, 2001) and the loss of flight in the kakapo (Livezey, 1992) and dodo (Livezey, 1993). Positive selection has also been invoked to explain island-specific phenotypes. For example, gigantism in oceanic tortoises is proposed to confer a selective advantage during the unpredictable long dry seasons on islands (Jaffe et al., 2011).

The amount of fixed and segregating deleterious mutations in Nothobranchius has been shown to correlate negatively with effective population size (Cui et al., 2019; Willemsen et al., 2019). Hence, we asked whether a similar trend occurred in nonannual species, such as the golden panchax (P. playfairii). The ratio of genes under relaxed selection detected with a phylogenetic method is lower than in genera within Nothobranchiidae, especially when compared to the annual genera. The relax method relies on fixed differences between species, which probably reflect the selective pressure in a more ancient past. Over a recent time frame, however, population size declined steadily over the past 200,000 generations in most of the golden panchax populations we analysed, probably reflecting the rising sea level and the shrinking island size since the last glacial period (Grant et al., 2012). Testing the accumulation of deleterious mutations using McDonald–Kreitman alpha metrics, we found evidence of deleterious mutations starting to segregate at low to intermediate frequencies, although probably not yet fixed, and hence probably preventing their detection by the phylogenetic method (which scores only the more frequent or fixed alleles). Compared to populations of annual killifishes of the genus Nothobranchius, individuals from Pachypanchax populations have lower levels of polymorphism, and yet they fixed fewer deleterious mutations. This is not surprising, however, because accumulation of deleterious mutations is not only a function of population size, but also relates to the selection regime, which eventually affects the shape of the distribution of fitness effects of new mutations. Golden panchax live in permanent water streams and are robust and long-lived fish, unlike Nothobranchius, which reside in ephemeral waters and are short-lived. Despite the declining population size, natural selection acting over a long evolutionary time may have further prevented life-shortening mutations from accumulating in this clade.

In agreement with recent findings that positive selection is in general difficult to detect especially in the presence of repeated relaxed purifying selection (Chen et al., 2020), we found weak evidence for positive selection in the golden panchax using both population genetics and phylogenetic methods.

The limited genetic diversity of the extant golden panchax populations, together with their limited distribution within the Seychelles archipelago and their progressively declining effective population size, furthermore represents a serious concern for the future survival of this species, and calls for effective conservation measures aimed at long-term preservation of this species.

4.3 | Relaxed selection in Pachypanchax affects embryonic developmental gene pathways

Despite relatively fewer genes having undergone relaxation of purifying selection in Pachypanchax compared to annual killifishes, they still amount to hundreds. In annual killifish genera from continental Africa, genes under relaxed selection are related to longevity and are differentially expressed between young and old fish (Cui et al., 2019). In contrast, genes under relaxed selection, as well as accelerated noncoding elements in Pachypanchax, are significantly enriched for early embryo development genes. An intriguing scenario compatible with developmental genes evolving under relaxed selection in Pachypanchax is that relaxation of selection could occur in both regulatory regions and coding regions of genes related to embryo diapause. If diapause were an ancestral trait to both annual and nonannual killifishes, once the ancestor of Pachypanchax colonized permanent water streams, they might have lost the selective pressure to remove any deleterious mutations affecting diapause-related developmental genes. Interestingly, although we did not detect extensive positive selection or highly enriched GO terms in candidate positively selected genes, the GO terms with an FDR cutoff from 0.05 to 0.2 for genes under positive selection are also related to developmental processes (cell cycle and semaphorin receptors). This could suggest that remodelling of early developmental processes may have occurred in the ancestors of Pachypanchax, involving both relaxation and positive selection for the same gene pathways. We provide a list of positively selected sites as candidate targets for future studies in Table S11. Hence, by detecting relaxed selection in a nonannual killifish clade that lost embryonic diapause, we may help reveal the genetic architecture of this unique trait that characterizes annual killifishes and that is lost (or masked) in nonannual species, such as those of the genus Pachypanchax.

Our results indicate that golden panchax probably colonized the Seychelles via transoceanic dispersal and support that prolonged insularism led to progressive population size reduction. Relaxation of purifying selection in golden panchax appears to be markedly distinct from the pattern observed in annual killifishes from continental Africa, largely affecting developmental, rather than ageing-related, genes.
CONCLUSIONS

Salt water barriers represent a considerable limit to the diffusion of freshwater taxa. Our analysis of the branching pattern as well as our molecular dating results suggest that at least in Cyprinodontiforms, transoceanic dispersal offers a likely mechanism to explain the current biogeographical pattern, and is supported by specific physiological adaptations (embryo development in seawater). We show that the steady decline in the effective population size may have caused recent relaxed selection leading to deleterious variants segregating in the population. In permanent water, relaxed selection does not preferentially target ageing-related pathways. Instead, relaxed selection targets both protein-coding and noncoding regions related to embryonic development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

D.R.V., S.V. and R.C. conceived and designed the study. S.V. and E.H. collected fish samples. Z.M. and R.C. made the sequencing libraries. A.T. performed seawater embryo incubation. S.P. and R.C. collected fish samples. Z.M. and R.C. made the sequencing library.

DATA AVAILABILITY STATEMENT

Raw resequencing reads are deposited at NCBI SRA Bioproject no. PRJNA702252. Computer programs are deposited at https://github.com/melop/killigenomics/tree/master/pachypanchax/. Variant calls, genome sequences and survival data are deposited at Mendeley data http://dx.doi.org/10.17632/vn4h8gg4z1.2

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