

Clonal polymorphism and high heterozygosity in the celibate genome of the Amazon molly

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The extreme rarity of asexual vertebrates in nature is generally explained by genomic decay due to absence of meiotic recombination, thus leading to extinction of such lineages. We explore features of a vertebrate asexual genome, the Amazon molly, *Poecilia formosa*, and find few signs of genetic degeneration but unique genetic variability and ongoing evolution. We uncovered a substantial clonal polymorphism and, as a conserved feature from its interspecific hybrid origin, a 10-fold higher heterozygosity than in the sexual parental species. These characteristics seem to be a principal reason for the unpredicted fitness of this asexual vertebrate. Our data suggest that asexual vertebrate lineages are scarce not because they are at a disadvantage, but because the genomic combinations required to bypass meiosis and to make up a functioning hybrid genome are rarely met in nature.

Asexual lineages present a paradox to biology. Overwhelmingly, theory predicts that asexual reproduction would have several main disadvantages. First, the classical model of demise, Muller's ratchet^{1,2}, states that deleterious mutations cannot be purged without meiosis, and their accumulation will lead to genomic decay and eventually extinction^{3,4}. Thus, obligate asexuals are predicted to be evolutionarily short-lived: a strictly clonal vertebrate population is unlikely to survive more than 10^4 – 10^5 generations in the face of incessant mutational pressure⁴. Second, the Red Queen hypothesis^{5,6} predicts that an absence of meiosis and formation of new genotypes in the zygote hinders the creation of genetic diversity, which is a precondition for adaptation to changes in the physical and biological environment. Third, recombination can uncouple beneficial and deleterious mutations, allowing selection to proceed more effectively with sex than without⁷. On the other hand, eukaryotic parthenogenetic lineages are all-female. Because they do not have to produce males, 100% of their offspring contribute to population growth, giving them a two-fold reproductive rate compared with sexual propagation⁸. However, all theories agree that the

disadvantages of asexual propagation quickly outweigh this advantage, and that clonality should eventually lead to extinction^{3,9}. Mixed support for this prediction exists: although some asexual species, for example, the obligate asexual waterflea, show deleterious mutation accumulation and are evolutionary extremely short-lived¹⁰, other asexuals are older than predicted and successful colonizers in their natural habitats^{11–14}.

Clonal lineages are numerous amongst unicellular eukaryotes¹⁵, plants¹⁶ and invertebrates^{17,18}, but vertebrates were long thought to be unable to exist as asexuals. However, in 1932, the Amazon molly, *Poecilia formosa*, was the first unisexual vertebrate to be described¹⁹, followed by the discovery of more than 50 naturally occurring fish, amphibian and reptile species, and more than 50 others that at least occasionally reproduce clonally²⁰. *P. formosa* became one of the paradigmatic cases that appear to violate the age predictions of Muller's ratchet and the dynamics of the Red Queen hypothesis. It is a highly successful colonizer of diverse habitats over a wide geographical range and mitochondrial DNA-based estimates postulated a much longer existence, exceeding the theoretical extinction time^{10,12,14}.

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As with most vertebrate asexuals, *P. formosa* shows features of an interspecific hybrid from distantly related sexual species, predicted to be an Atlantic molly (*P. mexicana*) mating with a Sailfin molly (*P. latipinna*)^{14,21}. Its mode of reproduction is gynogenesis²², an elaborate form of parthenogenesis where sperm from males of sympatric sexual species is ‘stolen’ (kleptosperry) to trigger embryonic development from unreduced diploid eggs (Fig. 1a). The sperm DNA is usually excluded from the developing egg; thus offspring are true clones of their mothers¹¹. In this study we investigate the ancestral history of the *P. formosa* genome and reveal its novel features.

Results

Genome assembly and gene annotation. To understand how a vertebrate genome evolves when the maternal genome is simply copied from generation to generation we sequenced a single *P. formosa* female. Total sequence coverage of 95-fold produced an assembly (Poecilia_formosa-5.1.2) with N50 contig and scaffold lengths of 57 kb and 1.57 Mb, respectively (Supplementary Tables 1 and 2). Two independent sets of protein coding genes (Ensembl: 23,615; NCBI: 25,474) were produced from the assembly. Both sets of genes were used throughout these analyses.

To measure the parental genome contributions to *P. formosa* we assembled *P. latipinna* and *P. mexicana* genomes using mostly assisted alignment²³ to the Poecilia_formosa-5.1.2 reference (*P. latipinna*-1.0 and *P. mexicana*-1.0) to total sizes of 815 and 803 Mb, respectively (Supplementary Tables 1 and 2). The number of protein-coding genes for each was similar to *P. formosa*: 25,220 for *P. latipinna* and 25,341 for *P. mexicana*. For population-level analyses we sequenced the genomes of an additional 19 *P. formosa*, 5 *P. latipinna*,

and 4 *P. mexicana* collected from various locations over each species’ range (Fig. 1b, Supplementary Table 3).

Transposable element history and activity. The absence of meiosis has been hypothesized to impact transposable element (TE) colonization of the genome. Theories predict that TEs should be either very few in number or absent from the genomes of asexuals because new integrations after zygote formation cannot occur and existing ones decay²⁴; or that TEs accumulate at unrestrained rates after the emergence of asexuality, because as the genome is subject to Muller’s ratchet they cannot be eliminated through recombination²⁵. This could eventually lead to extinction of asexual lineages. We detected no relevant difference in TE composition (Supplementary Note 1, Supplementary Tables 4 and 5) and transposition history between the genomes of *P. formosa* and the parentals (Fig. 2a). Most superfamilies are rather ancient and expanded long before the origin of *P. formosa*. The presence of TE sequences in the transcriptome indicated that some TEs are still active (Supplementary Note 1, Supplementary Table 6), in particular Gypsy elements (Fig. 2b), supported by genomic evidence for post-hybridization transposition events in *P. formosa* (Supplementary Note 1).

In summary, TEs show none of the predicted consequences of their host genome reproducing in the absence of recombination, but surprisingly look much like the original parental genomes and more broadly like other related teleosts. No increase in the TE load was detected in asexual arthropod lineages, including the water flea²⁶. In the genome of the asexual rotifer *Adineta vaga* a rather low percentage of TEs was found¹⁷, which seems to be mainly shaped by an ongoing process of TE acquisition by horizontal gene transfer and the predicted loss of ancestral elements. In *P. formosa* we detected

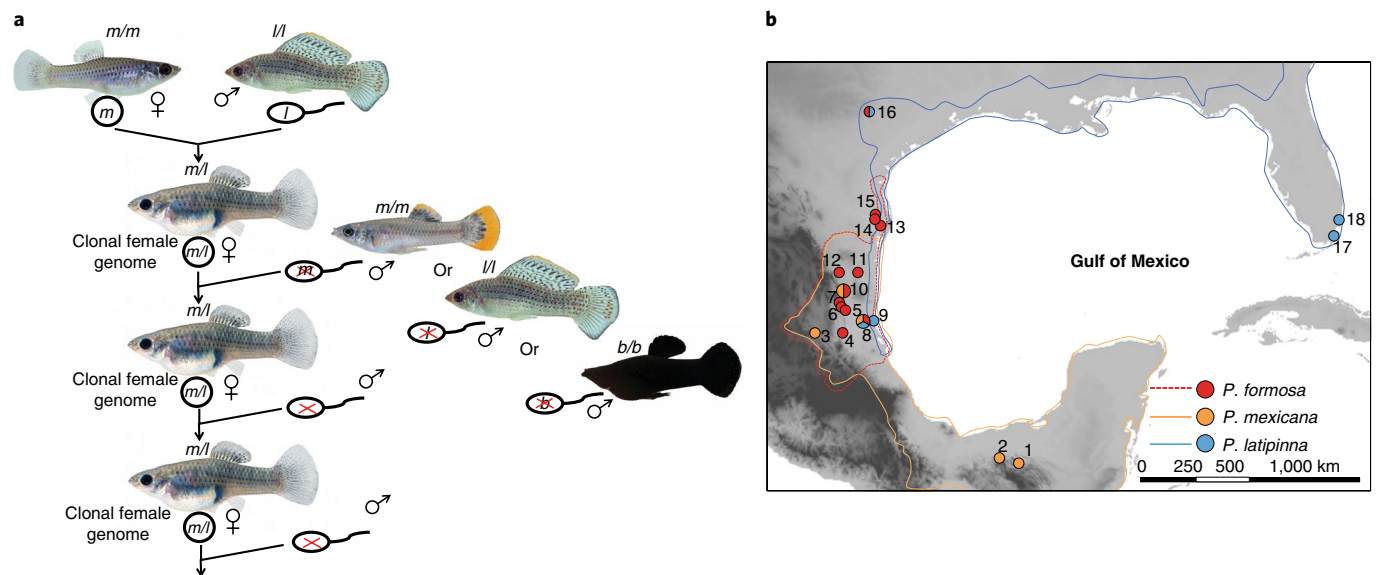


Fig. 1 | Reproduction schema of *P. formosa* and geographic origins of *P. formosa*, *P. mexicana* and *P. latipinna*. **a**, Through hybridization of a female *P. mexicana* (genome composition *m/m*) and a male *P. latipinna* (*l/l*), a hybrid (*m/l*) was produced that continued to reproduce through gynogenesis. Sperm from males of either one of the parental species (*P. mexicana*, haploid *m* genome, or *P. latipinna*, haploid *l* genome) or another sympatric species, *P. latipunctata*²², is used to trigger parthenogenetic development of the diploid oocyte (*m/l*), but the genetic content of the sperm is excluded (red cross) from the oocyte. In the laboratory, other *Poecilia* host males, for instance the ornamental black molly (*b/b*), are used. **b**, Map of the sampling sites and origins of stocks of *P. formosa*, *P. mexicana* and *P. latipinna* specimens used in this study. Multicoloured circles indicate sympatry of two (location 10, 16) or all three (location 9) species. 1, Pme_Cav; 2, Pme_Azu; 3, Pme_Ver; 4, Pfo-Ta-1, Pfo-Ta-2, Pfo-Ta-3, Pfo-Ta-4; 5, Pfo_Ma; 6, Pfo_Vic-1, Pfo_Vic-2; 7, Pfo_Lim; 8, Pfo_Cha, Pla_Cha, Pme_Cha; 9, Pla_Tam; 10, Pfo_Gua, Pme_Gua; 11, Pfo_Pad; 12, Pfo_Bar-1, Pfo_Bar-2, Pfo_Bar-3; 13, Pfo_Br, Pfo_Br-1, Pfo_Br-2; 14, Pfo_D1; 15, Pfo_Olm, Pla_Olm; 16, Pfo_SM, Pla_SM; 17, Pla_Flo; 18, Pla_Fld. For location details see Supplementary Table 2. The natural range of the three species is indicated by the coloured lines. At location 16, *P. formosa* has been introduced by human activities. Map dimensions: ca. 102–78° W longitude; ca. 17–32° N latitude.

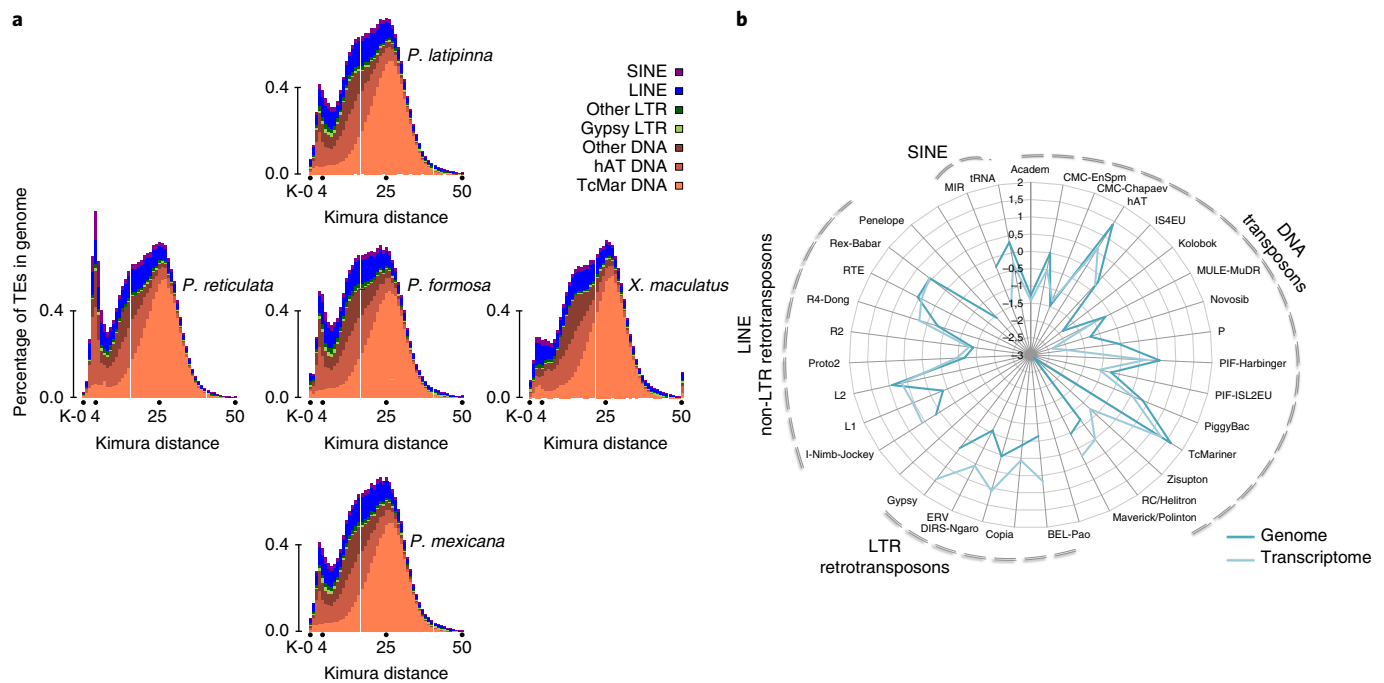


Fig. 2 | Evolutionary history and expression of TEs. a, Copy-divergence analysis of TE classes in five poeciliid genomes, based on Kimura 2 parameter distances. The percentages of TEs in genomes (y axis) are clustered based on their Kimura values (x axis; K values from 0 to 50; arbitrary values). Older copies are located on the right side of the graphs while recent copies are located on the left side. DNA transposons (TcMariner, hAT and all other DNA superfamilies) are shown in red tones. LTR retrotransposons (Gypsy and all others) are in green tones. LINE and SINE retrotransposons are in blue and purple tones, respectively. **b**, The proportion of TE superfamily representation in the genome and the transcriptome of *P. formosa*. The proportion of each TE superfamily was initially calculated as (% of TE superfamily \times 100) / total % of TEs in the genome or transcriptome, and then for the spider graph transformed to \log_{10} values. The expression of Gypsy elements might be the result of their activity rather than of general background expression because their relative fraction is notably higher in the transcriptome than in the genome.

no TE sequences, whose absence from the parental genomes would indicate horizontal gene transfer.

Gene evolution. The standard genome-wide analyses on gene evolution (positive selection, gene duplication and gene family expansion/contraction; Supplementary Note 2, Supplementary Fig. 1, Supplementary Tables 7–9) did not reveal any unusual features of the *P. formosa* genome in comparison with other teleosts that reproduce sexually. Genes associated with functions that are assumed to be non-essential and thus dispensable for asexual female reproduction, including genes pertinent to spermatogenesis and meiosis, showed no damaging variants (such as frameshifts, premature stops and high sequence divergence; Supplementary Table 10–12). Similarly, we find the average number of loss of function variants (LoFs) in *P. formosa*, compared to the parental species (Supplementary Note 2, Supplementary Table 13), to be slightly less. Moreover, LoF counts are all in the range of what has been reported for the genomes of related sexual Poeciliid species²³. Genome-wide analyses to detect genes showing signs of relaxation from purifying selection revealed seven such genes in *P. formosa*, but also seven in *P. mexicana* and 11 in *P. latipinna* (Supplementary Table 14). For the opposite phenomenon, positive selection, we also observe similar counts: *P. formosa* (24), *P. mexicana* (22) and *P. latipinna* (27) (Supplementary Table 15). In the genome of *P. formosa* a total of 211 non-processed or duplicated pseudogenes (not overlapping with LoFs) were recorded, similar to its sexual relatives (*P. mexicana* 268, *P. latipinna* 266, *P. reticulata* 278). In summary, asexual genic evolution of the Amazon molly is not obviously different from the sexual parental species.

Gene copy number variation (CNV) impacts genome evolution and adaptation²⁷. The extent of CNVs is not significantly different

between the Amazon molly and its parental species (Supplementary Note 2) and gene ontology (GO) term analysis (Supplementary Tables 16–21) revealed that several of these variants are most probably expansions of certain TEs.

Segmental duplications of genes linked in a common process can give hints on specific features of *P. formosa*. The asexual reproduction mode, apomixis, requires that diploid oocytes are formed without meiosis. Disturbance of meiosis leads to mis-segregation of chromosomes and can even turn meiosis I into a normal mitosis²⁸. Several genes involved in the meiosis-specific separation of homologous chromosomes show CNV in *P. formosa* compared with the parentals and other sexual species (Supplementary Note 3, Supplementary Table 22). In particular, genes for cyclin-dependent kinase 1, an essential regulator of meiotic kinetochore–microtubuli capture, and its oocyte-specific activator, Ringo/speedy, are present in multiple copies. We hypothesize that the expression imbalance brought about by such CNVs disturbs the proper establishment of kinetochore unipolarity in meiosis I and induces a mitotic division, thereby generating diploid, ameiotic oocytes.

Ancestry and evolutionary age of the Amazon molly. A low level of genomic decay in the asexual species could be expected if new lineages are repeatedly produced from hybridization of the parental species. Previous studies based on small fractions of mitochondrial or nuclear genomes showed conflicting origins as to a single¹⁴ or multiple F_1 hybrids or backcrosses^{14,29}.

We performed ancestry estimates for *P. formosa* using our high-quality single-nucleotide polymorphism (SNP) data set (292,324 sites). In total 53,175 sites display fixed nucleotide differences between the parentals, of which 47,359 were inferred to be heterozygous in *P. formosa* for the two parental alleles confirming an

F₁ hybrid origin. All *P. formosa* isolates consistently display a heterozygosity (*H*) index of 0.5 (Supplementary Table 23).

We then reconstructed the complete mitochondrial genomes of all samples. Haplotypes of *P. formosa* were more closely related to *P. mexicana* than to *P. latipinna* (Supplementary Note 4, Supplementary Fig. 2) confirming *P. mexicana* as the maternal ancestor. Phylogenetic trees (Fig. 3) revealed that all *P. formosa* are united in a highly supported single clade that is consistent with a single maternal (mitochondrial) origin. Using a time-calibrated Bayesian phylogenetic tree of mitogenomes (Supplementary Note 4, Supplementary Fig. 3) we estimate a minimum age for *P. formosa* of at least 100,000 years, exceeding by far the predicted age for extinction from previous calculations based on the threat from Muller's ratchet¹².

Gene conversion. Given a considerable age and single origin we looked for possible mechanisms that could affect the extent of the predicted negative consequences of asexual reproduction. One explanation could be that enhanced gene conversion reduces genetic decay. Homogenization of local sequence tracks through gene conversion will limit the spread of LoFs by exposing mutations to selection or overwriting them entirely. We find a rate of 3×10^{-8} conversion events per generation per site in *P. formosa* (Supplementary Note 5), which is in the range of other asexually reproducing species³⁰ but still two orders of magnitude lower than for sexual species^{31,32}.

Paternal introgression. Sustained genomic diversity and refreshing alleles damaged by the process of Muller's ratchet could come from exceptional uptake of paternal sperm DNA. Microchromosomes, which occur in some *P. formosa* individuals and are inherited like B-chromosomes^{33,34}, have been explained to be derived from incomplete removal of paternal DNA after insemination of *P. formosa* oocytes. This hypothesis critically depends on the assumption that microchromosomes are indeed of paternal origin and that they contain coding DNA. Hence, we measured non-meiotic introgression from host males, hypothesizing that within the *P. formosa* genome allele imbalance will exist in regions that experienced introgression due to additional copies present on the microchromosomes (Supplementary Note 6). We detected 19 putatively introgressed scaffolds across 5 *P. formosa* samples (Fig. 4, Supplementary Table 24). The total introgressed scaffold size ranged from 0.33 to 8.1 Mb, approximately 1% of the genome, adding up to hundreds of protein coding genes (with no obvious enrichment of GO terms; Supplementary Note 6, Supplementary Tables 25 and 26), microRNAs and other functional units from a recombining parental genome.

Hybrid genome constitution. One reason for the fitness and ecological success of *P. formosa* could be that heterozygosity of the interspecies hybrid is maintained in the asexual lineage, described in the 'frozen hybrid genome' hypothesis³⁵. Indeed, heterozygosity in the parental species was on average approximately tenfold lower than in *P. formosa* (Supplementary Table 27). Next we reconstructed haplotypes for all isolates to examine phylogenetic relationships (Supplementary Note 7). We observe two strongly supported clades, each consisting of the parental haplotypes and those *P. formosa* haplotypes derived from this parent (Supplementary Fig. 4), again supporting the single origin hypothesis.

Allele-specific gene expression analysis from three different organs of *P. formosa* revealed that only 1.2 to 4.1% of genes had an expression bias towards one of the parental alleles, whereas for the overwhelming majority of genes there is about equal contribution from both parental alleles (Supplementary Fig. 5). This demonstrates that *P. formosa* is not only a genomic but also a 'functional' hybrid.

To test if the increased level of heterozygosity in *P. formosa* might counter the predicted detriment under the Red Queen

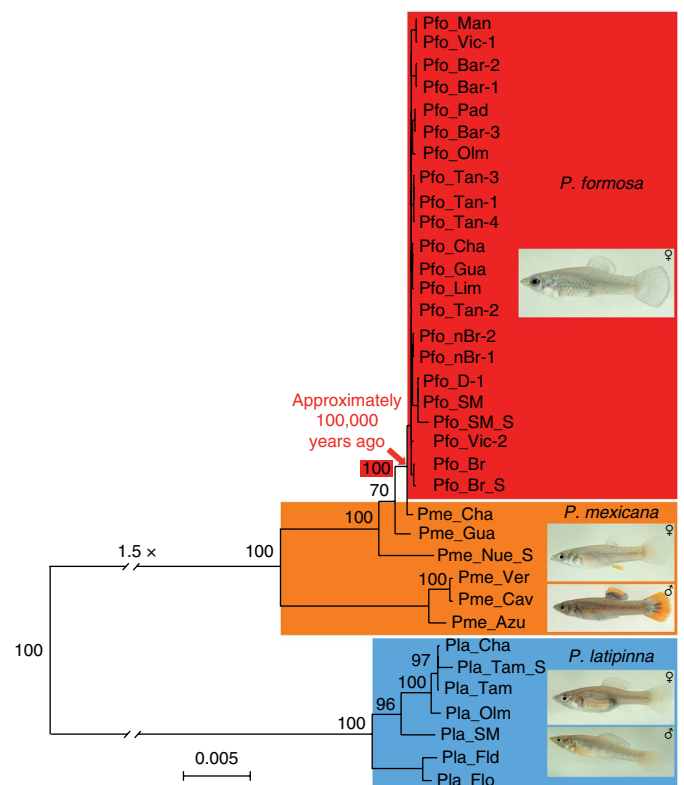


Fig. 3 | Evolutionary origin of *Poecilia formosa*. Maximum likelihood phylogenetic tree based on 35 complete mitochondrial genomes (16,587 bp), obtained with the program PhyML. Samples labeled ‘_S’ represent reference mitochondrial genomes. All other mitochondrial genomes were assembled from whole genome sequencing reads. The numbers above the branches represent bootstrap values based on 100 resampled data sets. The branching point for *P. formosa* from *P. mexicana* was estimated at about 100,000 years ago; for details see Supplementary Note 4 and Supplementary Fig. 3.

hypothesis, we looked at a system that in clonal organisms should be at major disadvantage, namely immune genotypic variability. The cell-mediated adaptive immune response is regulated by the major histocompatibility complex (MHC)³⁶. Variability in these multicopy genes is positively correlated with immune competence³⁷. Remarkably, we discovered high diversity in MHC class I genes (80 different alleles in 20 individuals) (Fig. 5a). MHC class II genes were less variable, but still 36 different alleles were found (Fig. 5b). MHC copy number in *P. formosa* is generally in the same range as in its sexual ancestors and in some clones even exceeded average levels (up to 13) with higher variation than expected from previous studies on a limited data set^{38,39}. However, one clone (F in Fig. 5a) had only two MHC class I genes, suggesting that some functional gene copies might have been lost in this lineage. We also examined 15 critical members of the innate immune system for variability. In the sexual parental genomes within-individual inter-allelic diversity was low, and for the majority only a single allele was retrieved. However, *P. formosa* always comprised at each gene locus two considerably different alleles (Supplementary Table 28, see also Fig. 6 for neighbour-joining amino acid trees of toll-like receptors). Alleles derived from one parent were similar among the *P. formosa* individuals and clearly of monophyletic origin. The genetic distance of immune gene sequences within individuals was significantly higher for *P. formosa* (Supplementary Fig. 6a). Overall, the immune system genes of *P. formosa* present an unexpected high level of genetic variability.

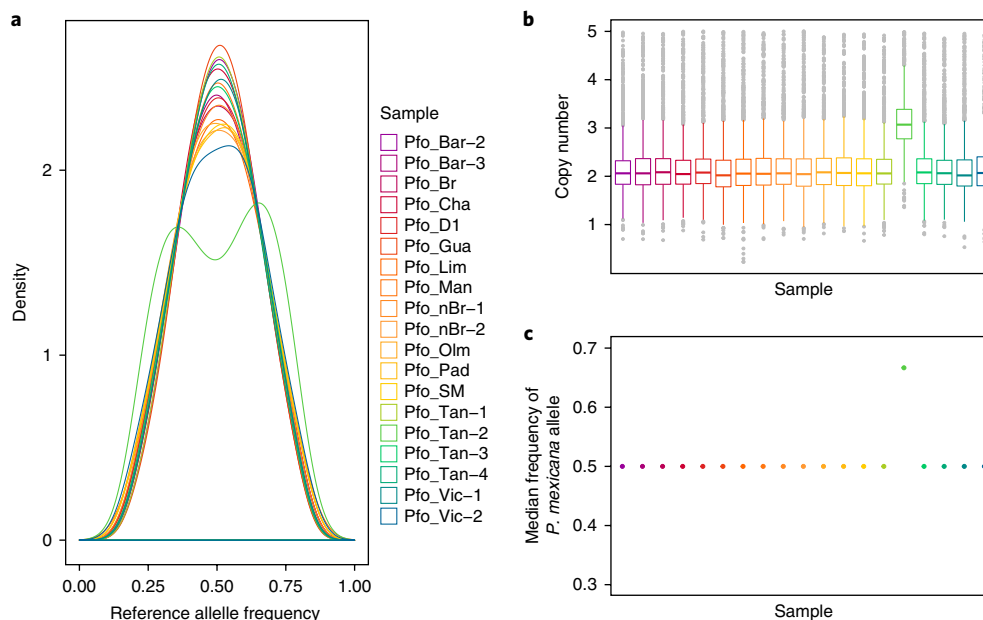


Fig. 4 | Introgression of paternal genomic elements. Evidence supporting paternal introgression of scaffold K1519701.1 into sample Pfo-Tan-2.

a, Reference allele frequency distribution at called heterozygous sites of scaffold K1519701.1. In the absence of introgression, the reference allele frequency at heterozygous sites (density) follows a normal distribution centred at 0.5. The distribution of sample Pfo-Tan-2 deviates from the theoretical normal distribution centre, and instead presents two peaks at frequencies of ~ 0.33 and ~ 0.66 , which is consistent with the presence of 2 copies of scaffold K1519701.1 that originate from one parental species and 1 copy of scaffold K1519701.1 from the other parental species. **b**, Copy number distributions across 1 kb repeat-free windows in scaffold K1519701.1. All samples except Pfo-Tan-2 show a copy number distribution that is centred at 2, suggesting that they are diploid. The distribution of Pfo-Tan-2 is centred at a copy number of 3, indicating it is triploid at this scaffold. **c**, Median frequency of *P. mexicana* alleles at heterozygous sites. For diploid organisms, the frequency of any allele at heterozygous sites should be centred at 0.5. However, for sample Pfo-Tan-2, the frequency of the *P. mexicana*-derived allele at heterozygous sites is 0.66, indicating that the introgressed scaffold K1519701.1 in Pfo-Tan-2 was derived from *P. mexicana*. The legend applies to all panels.

The immune genes also demonstrate that genetic variation within *P. formosa* is not restricted to intra-individual heterozygosity but that one and the same parental gene copy exists in many alleles. The phylogenetic trees obtained for each of the immune genes indicate a single origin for each group of the parental alleles. Consequently, such differences between alleles must be due to mutations. Nucleotide sequence differences in the open reading frame were generally much lower than in the noncoding regions (Supplementary Fig. 6b). With the exception of *CD59*, which is under positive selection in all three species, most immune genes are under strong purifying selection (Supplementary Table 29).

Discussion

Analysis of the Amazon molly genome and comparison to its sexual parental species uncovered unanticipated features that may change our view on asexual organisms that practice gynogenesis. Unexpectedly, we found no widespread signs of genomic decay. This is not explained by a recent origin because our age calculations of about 100,000 years from whole mitochondrial and nuclear genome data substantiate earlier estimates of 120,000–280,000 years¹¹. Thus, given a generation time of 3–4 months, *P. formosa* has existed for approximately 500,000 generations, and has survived several-fold beyond its Muller's ratchet-based predicted extinction¹².

Despite such an ancient origin we find genes that serve organs or processes that are no longer in use in the all-female fish, such as spermatogenesis, male development and meiosis genes, are not corrupted. The Mexican tetra, which inhabits a similar natural range to *P. formosa*, has evolved cave populations from surface fish, some not older than 30,000 years⁴⁰, yet all show total loss of organs no longer in use, such as eyes. A similar trait loss situation could

have been expected in the Amazon molly, but was not observed. Another simple explanation for the much lower level of genomic decay than predicted from mathematical models may be that not enough time has elapsed. In this case the Amazon molly genome sets a new time point for what is 'not old enough' for a vertebrate genome to undergo genetic degeneration. This result also has implications for 'regressive' vertebrate systems like the cavefish as it provides a baseline for how many generations have passed without any signs of neutral morphological and genetic degeneration or regression appearing. It could make an argument that comparatively fast trait loss in the cave environment is based on selection and standing variation in cavefish.

Another explanation is introgression of DNA into an asexual lineage that represents a unidirectional flow of genetic material that can compensate for harmful alleles accumulated in the genome. In the ancient asexual bdelloid rotifers occasional parasexual transfer of genetic material between individuals, known as horizontal gene transfer in bacteria, generates divergence with up to 10% of genes of putative non-metazoan origin⁴¹. Unisexual salamanders of the *Ambystoma jeffersonianum-laterale* complex occasionally interrupt clonality by 'stealing' paternal DNA from sympatric sexual species (kleptogenesis) and loss of part of the clonal genome^{20,42}. In *P. formosa* our sequence-level analysis detected 'genetic addition' as the mode for introgression of subgenomic amounts of DNA in about 25% of the samples at a much higher resolution than is possible with cytogenetic methods, suggesting paternal introgression occurs more frequent than initially thought. These introgressed sequences are derived from genic regions of the paternal genome.

Most evaluations of Muller's ratchet use estimates of important parameters, such as mutation rate and population size, which are

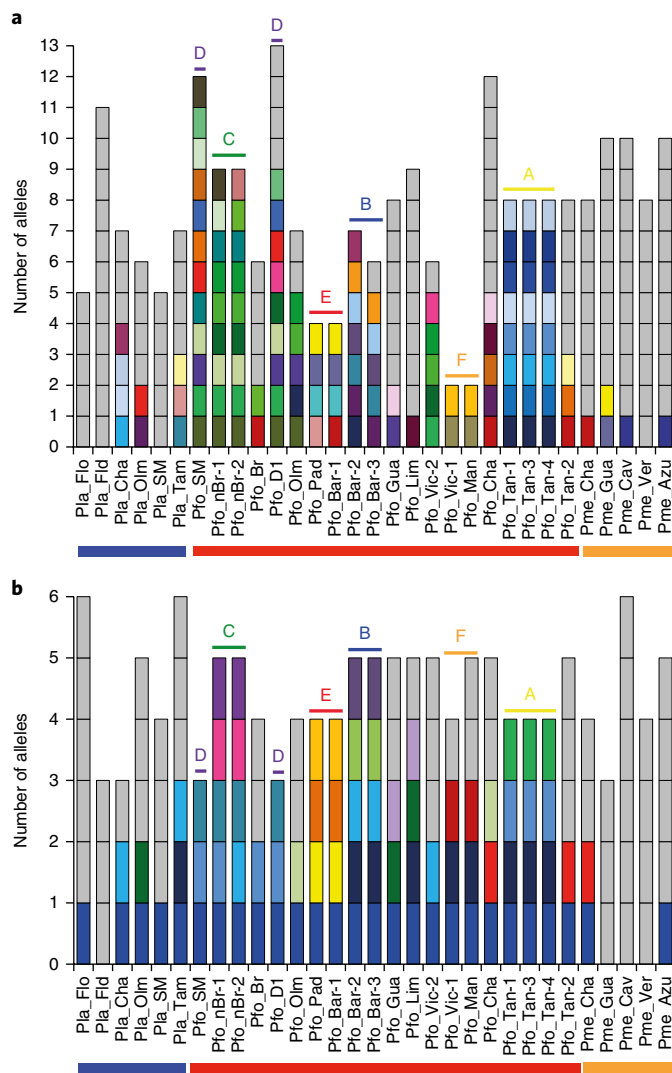


Fig. 5 | MHC variability in different Amazon molly clones and field sites. **a,b.** For MHC class I (**a**) and MHC class II (**b**) gene alleles we plotted individuals that are sorted geographically from north to south on the x axis. Unique alleles are shown in grey, the coloured bars depict alleles that were found in more than one individual. Coloured letters indicate the mitochondrial haplotypes that were found in more than one individual (see Supplementary Fig. 2). Bars indicate species: blue, *P. latipinna*; red, *P. formosa*; orange, *P. mexicana*.

unknown for *P. formosa*. Lower actual mutation rates and higher numbers of individuals might contribute to the discrepancy between the expected and observed rates of genomic decay. Although such differences from assumed values are difficult to evaluate, our genomic data now provide indications about processes that can affect Muller's ratchet. Introgression has not been considered in calculations of the expected time to extinction (T_{ex}) for asexual species, including *P. formosa*. We recalculated predicted extinction times for the Amazon molly according to a published method¹², but considered relief from genomic decay when different amounts of genetic material are introgressed from sexual parental species at the frequency observed in our data. This resulted in a slight increase in T_{ex} (Supplementary Fig. 7). However, paternal introgression alone does not provide sufficient explanation for how long *P. formosa* has outlived its predicted extinction time. Other genomic features of *P. formosa* that are more difficult to quantify are traces of past events of loss of heterozygosity. It has been concluded that loss of

heterozygosity can have a similar beneficial effect to segregation during meiosis⁴³, and consequently could also counteract the ratchet.

The Red Queen hypothesis is often touted as an explanation for why sexual reproduction persists, as it posits that recombination allows species to maintain genome diversity against ecological stressors, such as pathogens. Without recombination, how do rare asexual vertebrate species remain extant? We propose that the available standing genetic variation is a sufficient starting point. The evolutionary advantage of such hybrid vigour has been shown in hybridogenetic frogs⁴⁴ and it is noteworthy that all asexual vertebrates are of hybrid origin²⁰. Our results provide support for earlier predictions that being a 'frozen hybrid'⁴⁵ with elevated heterozygosity provides a fitness advantage (assuming polymorphisms improve fitness) and a possible resource for responses to natural selection. Moreover through de novo mutations new clones can arise from this original hybrid genome.

Interacting effects of parasites and the accumulation of mutations were shown by computer simulation to enhance Muller's ratchet in a population that experiences Red Queen dynamics⁴⁶. Our genome analyses uncovered—in contrast to predictions of the Red Queen model for asexuals—a high genomic diversity. Field studies revealed that *P. formosa* does not have a higher parasite load than sympatric sexual species⁴⁷. We therefore assume that the ratchet for Amazon mollies clicks at a lower speed than projected under the severe conditions of the hypothesis.

We propose that genetic diversity between clones offers at minimum a short-term benefit to the asexual species in coping with environmental challenges. Those clones that acquired new adaptive mutations will thrive, while others that are less fit, like the one with only two MHC class I genes, will disappear. On this basis, we posit that in the absence of recombination *P. formosa* can evolve by clonal selection of naturally occurring mutations and competition between clones. Because Amazon mollies do not have to pay the 'two-fold costs of sex'⁴⁸, they have an increased population growth rate and can more quickly reach large population sizes^{12,14}. Sperm-dependent parthenogens hinge on their ecologically similar sexual host but exhibit some niche separation, as they otherwise are expected to out-compete them and ensure their own demise⁴⁸. In large populations of *P. formosa* multiple clones can be maintained, favouring selection of advantageous clones and clearing of less fit ones.

All known obligate asexual vertebrates are hybrids and we provide evidence that a hybrid genome might be one driver of fitness of asexual lineages. There is increasing evidence that interspecific hybridization is much more frequent than previously thought; it is estimated that approximately 10% of animal species hybridize regularly with at least one other species⁴⁹. Given that even after 500,000 generations in the absence of recombination the Amazon molly does quite well, one might ask why clonal vertebrates are so rare, and in particular why *P. formosa* arose only once despite the continuing co-occurrence of the parental species? So far, all attempts at a laboratory synthesis of *P. formosa* by crossing the parental species have failed^{33,50}. Combining just the right genotypes may have been key to allow asexual reproduction to occur. In interspecific hybrids, certain combinations of parental genes can lead to Dobzhanski–Muller incompatibilities, thus many hybrid genotypes will not be favourable for generating new species^{51,52}. In addition, finding the right combination of immune genes might be a problem. While immune gene diversity is important, too much diversity can be disadvantageous by either hindering effective pathogen detection^{53,54} or triggering autoimmune diseases⁵⁵. In *P. formosa*, however, no evidence of reduced pathogen resistance or autoimmune disease is found, indicating that their highly diverse immune genes are compatible^{47,56}.

Taken together, we favour a 'rare formation hypothesis' specifying that clonal species might not be rare because of their inferiority

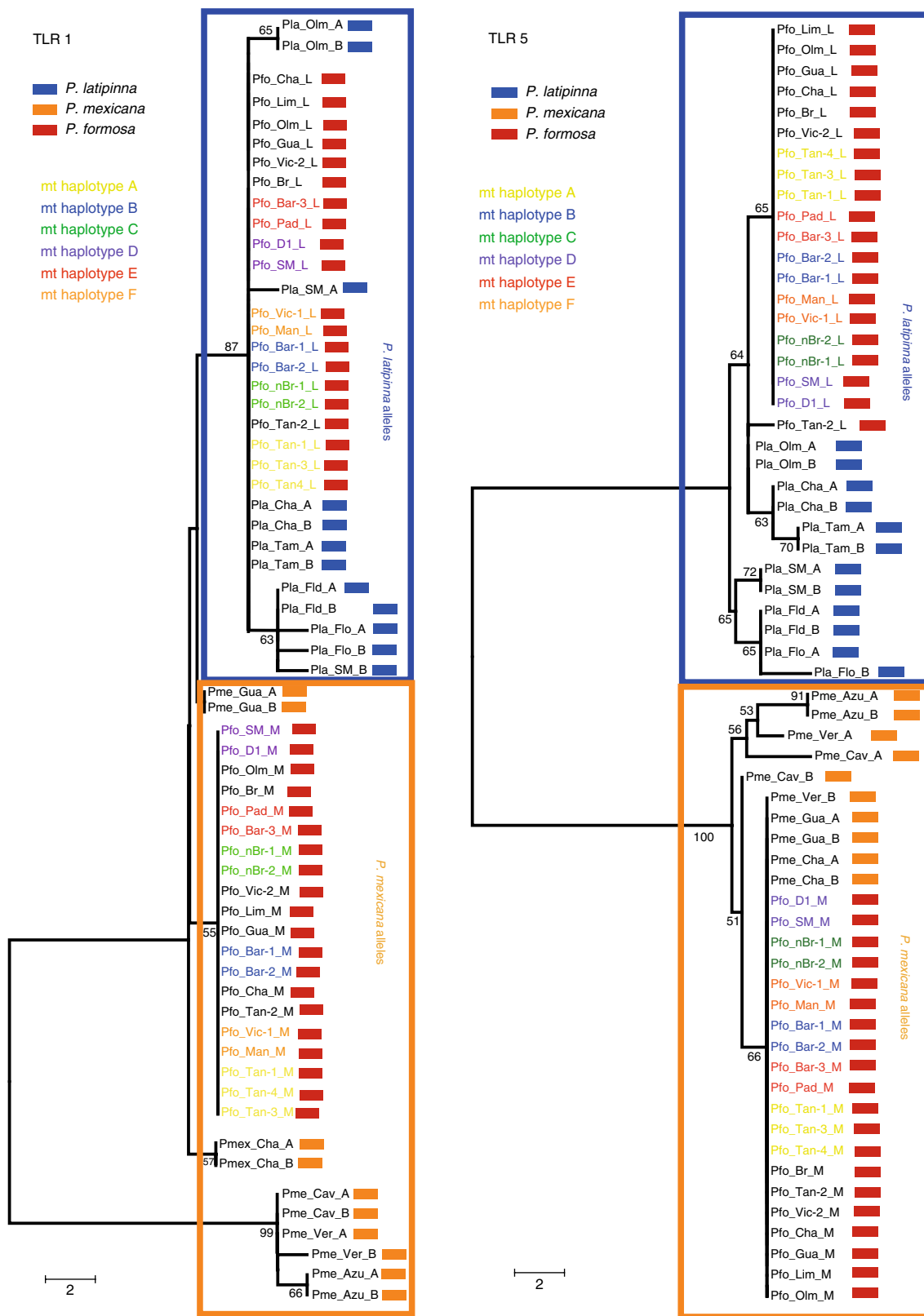


Fig. 6 | Phylogenetic reconstruction of toll-like receptors TLR 1 and TLR 5. Neighbour-joining trees (unrooted) are shown based on the amino acid sequences of the largest open reading frame of innate immune genes TLR 1 (802aa) and TLR 5 (759aa). Only bootstrap support values >50% are shown (1,000 randomizations). Species are marked by coloured squares after the sample names. The colour used for the names corresponds to the mt haplotype (see Supplementary Fig. 2; black, individual haplotype). For both TLRs only very few individuals from each of the parental species showed two different alleles, whereas each *P. formosa* individual always has two clearly divergent alleles (L indicates *P. latipinna* origin, M indicates *P. mexicana* origin), reflecting the hybrid origin of the species.

to sexual species, but because the genomic combinations that allow successful survival and reproduction are very specific¹⁴.

Methods

Biological material. The fish used in this study from aquaria housed stocks were kept and sampled in accordance with the applicable European Union and national German legislation governing animal experimentation. We hold an authorization (568/300-1870/13) from the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law (TierSchG).

Sequencing and assembly. The *P. formosa* DNA for Illumina shotgun sequencing was derived from a single adult female (Pfo_Bar-1) from a clonal line established from a fish collected in 1996 in the Rio Purification near Barretal, Tamaulipas, Mexico. Total sequence genome coverage on the Illumina HiSeq 2000 instrument was ~95× with tiered library insert sizes of pre-determined sequence coverage for each (45× fragments, 45 × 3 kb, 5 × 8 kb and 0.05 × 40 kb). All sequences were assembled using ALLPATHS³⁷ with default parameters. Assembly connectivity was further improved with the external scaffolding tool SSPACE³⁸ and final scaffold correction was achieved with mate pair (3 kb) discordance analysis using REAPER³⁹. The total assembled bases comprise 748 Mb.

For a single male *P. latipinna* from north of Tampico (Pla_Tam) we generated 34× sequence coverage of paired 100 bp reads (20×) and 3 kb paired reads (14×) and for *P. mexicana* (single female from Laguna Champaxan, Pme_Cha) 30× sequence coverage of paired 100 bp reads (22×) and 3 kb reads (8×). Both species-specific sequence sets were aligned to the PoeFor_5.1.2 reference to generate sequence contigs as previously described²³. Additional scaffolding was achieved with the use of SSPACE³⁸. The assemblies comprise 18,161 and 18,275 total scaffolds with N50 contig and scaffold lengths of 33 kb and 280 kb, respectively, for *P. latipinna*, and 40 kb and 270 kb, respectively, for *P. mexicana*.

For all *P. formosa* population samples we generated sequence on the HiSeq2500 instrument (100 bp read lengths) whereas the *P. latipinna* and *P. mexicana* isolates were sequenced on Illumina ×10 instruments (125 bp read lengths). All reads were pre-processed by removing duplicate reads with Picard v.1.113 (<http://picard.sourceforge.net>), and only properly paired reads were aligned with the appropriate reference using BWA-MEM⁴⁰.

Gene annotation. Automated gene predictions followed previous methods for Ensembl⁶¹ and NCBI⁶² pipelines, including masking of repeats before *ab initio* gene predictions, for evidence-supported gene model building. However, the *P. latipinna* and *P. mexicana* genomes were only annotated for gene content with the NCBI pipeline⁶². In both annotation processes gene models were supported or novel based on RNA sequencing (RNA-seq) data from *P. formosa*, *P. mexicana* and *P. latipinna* independent tissues (see NCBI annotation report: https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Poecilia_formosa/101/) for the full list of tissues. For the Ensembl *P. formosa* gene build only the RNA-seq data from liver, brain, skin ovary, gills and embryonic tissues were used. The final Ensembl *P. formosa* gene set comprises models based on orthologous proteins from the vertebrate division of UniProtKB, longest translations of some stickleback gene models from Ensembl 73, as well as models from RNA-seq data. RNA-seq data were used to further improve gene model accuracy by alignment to nascent gene models to delineate boundaries of untranslated regions as well as to identify genes not found through interspecific similarity evidence from other species. Our measures of gene representation for the aligned core eukaryotic genes ($n = 458$) using CEGMA⁶³ showed >92% were complete at 90% of their estimated length in all three *Poecilia* species.

SNP and heterozygosity analysis. Resequenced genomes were aligned against the *P. formosa* reference to extract SNPs with SAMtools and VCFtools using stringent criteria. Fixed heterozygous sites informing about ancestry were defined by the rule that the heterozygous genotype should be observed in all isolates and in case of missing genotypes at least 5 *P. formosa* isolates are heterozygotes with the remaining isolates missing genotype calls. Genome-wide expected heterozygosity (theta) was estimated using a maximum-likelihood method⁶⁴ based on sites with a minimum of 4× sequence coverage after taking into account sequencing error and random sampling of two homologous chromosomes in a diploid organism.

The within-species per-site nucleotide diversity was calculated for sequenced genomes of each species using VCFtools⁶⁵ and was averaged over all the SNP sites. Tajima's *D* values were calculated for each 50 kb non-overlapping genomic window using genome-wide SNP data sets in the software VCFtools, and subsequently a genome-wide average was calculated.

To validate a previous study that claimed backcrossing has occurred we used INTROGRESS as in ref.²⁹ to analyse the introgression of genotypes between divergent, hybridizing lineages, including estimating genomic clines from multi-locus genotype data and testing for deviations from neutral expectations.

DNA sequences described in population sequencing for *P. formosa*, *P. latipinna* and *P. mexicana* from 5 individuals were each aligned with the homologous reference of each at an average input sequence coverage of 11× using BWA-MEM.

Total SNPs were called by GATK HaplotypeCaller and GenotypGVCFs⁶⁶. All SNPs were used to report expected LoFs classified as single-base substitutions that disrupt splice acceptor, splice donor, stop loss or gain codons using the VAAST software⁶⁷. To filter variants that were shared across all samples in either of the sexual parents we removed LoFs that were shared in all. We reasoned the likelihood of fishes having LoFs fixed among different populations is very rare. However, we did allow LoFs to be shared across all in the *P. formosa* samples. As they are clonal it is feasible that the LoFs could remain fixed over many generations.

Mitochondrial genome analyses. Reference mitochondrial genomes (of all three species were produced from long-PCR products sequencing (Supplementary Note 4). Long PCRs were performed with primers designed from the complete mitochondrial genomes of *Hypoatherina tsurugae* (NC_004386.1), *Gambusia holbrooki* (NC_004388.1), *Melanoteania lacustris* (NC_004385.1) and *Colalabis* (NC_003183.1). Fragment 1 (10.7 kb): TRPFishFor: 5'_AGACCAAGGGCCCTCAAAGCC_3', 15995Rev. Fish: 5'_CTTTGGGAGCTAGGGGTGAGAGTT_3', Fragment 2 (7 kb): L15995: 5'_AACTCTCACCCCTAGCTCCCAAAG_3', TRPFishRev: 5'_GGCTTTGAAGGCCCTTGGTCT_3', Fragment 3 (7.3 kb): H16100R: 5'_ATGTAGGGTTACAYTACTTTAAAT_3', ATP6fPoc-long: 5'_AACTATCWATTAACATAGGTCTTCGWGGCGCT_3' using Takara Taq under the following conditions: 94°C 15 s, 94°C 15 s, 49 to 63°C 30 s followed by 68°C 6.45 min for each step, 72°C 12 min.

PCR products were mechanically hydro-sheered and cloned into shotgun libraries before Sanger sequencing. Sequences were first assembled into contigs using the phred/phrap assembler (www.phrap.org/phredphrapconsed.html) and then contigs further merged using the cap3 assembler (<http://seq.csiastate.edu/cap3.html>). Remaining gaps were closed by additional PCRs. Whenever aligned shotgun sequences indicated potentially conflicting sequence data, fragments were re-amplified and re-sequenced by specific direct PCRs. The mitochondrial genomes from NGS data were assembled with the Geneious program version 8.1.7 (<http://www.geneious.com/>) (map to reference algorithm, medium/low sensitivity, up to 5 iterations) using the *P. reticulata* mitochondrial genome (Genbank Accession number AB898687.1) as outgroup reference. Consensus sequences were edited with Bioedit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and analyzed using MEGA (genetic distances) (<http://www.megasoftware.net/>) and PopART (mitochondrial haplotype minimum spanning network) (<http://popart.otago.ac.nz>).

Repeat analyses. Repeat (TEs and non-TEs) libraries of *P. formosa*, *P. mexicana* and *P. latipinna* were established using an automatic annotation with RepeatModeler1.0 (<http://www.repeatmasker.org>), combined with manual search of known TE proteins and phylogenetic classification. For comparison we used the genomes of platyfish (*Xiphophorus maculatus*)⁶⁸ and guppy (*P. reticulata*) (http://www.ncbi.nlm.nih.gov/assembly/GCA_000633615.2). TE superfamily classification is based on a universal classification⁶⁹. TE contents in genome assemblies were estimated using RepeatMasker 3.3.0 (<http://www.repeatmasker.org>). The relative age of TE copies in poeciliid genomes was calculated through Kimura distance analyses (<http://www.repeatmasker.org/genomicDatasets/RMGenomicDatasets.html>). The proportion of sites with transitions (*p*) and transversions (*q*) were transformed as follows: ($K = -\frac{1}{2} \ln(1 - 2p - q) - \frac{1}{4} \ln(1 - 2q)$). Transcriptome assembly of the Amazon molly was masked to evaluate the proportion of transcribed TE superfamilies.

For phylogenetic analyses, nucleotide sequences were first translated using Augustus⁷⁰. TE proteins (reverse transcriptase or transposase) were then aligned using MUSCLE⁷¹ in PhyML package⁷² and phylogenetic trees were reconstructed with the maximum likelihood method using approximate likelihood ratio test (aLRT) non-parametric branch supports.

Gypsy insertions longer than 3 kb (based on RepeatMasker annotation) were manually analysed to identify specific insertions. Insertions were extracted with flanking regions (+/- 1 kb) and aligned against *P. mexicana* and *P. latipinna* genome assemblies via BLAST. *P. formosa* insertions were considered specific when they shared no flanking regions with the parental species. A similar method was used to investigate the presence of solo-LTR.

Gene selection. Orthologous genes of *P. formosa*, *P. latipinna*, *P. mexicana* and *P. reticulata* were identified using Inparanoid <http://inparanoid.sbc.su.se/cgi-bin/index.cgi> (default settings). For each gene both protein and cDNA sequences were aligned using clustal-omega (v.1.2.1) <http://www.clustal.org/omega/> (option: -outfmt fasta). Non-conserved blocks from the alignments were removed by Gblocks (v.0.91b) <http://molevol.cmima.csic.es/castresana/Gblocks.html> (options: -b4 10 -b5 n -b3 5). The resulting sequence alignments were converted into a codon alignment using pal2nal (v.14) <http://www.bork.embl.de/pal2nal/>. Codon alignments were converted into phylip format using clustal-omega (option: -outfmt phy). Trees were built using Phylip (v.3.696, <http://evolution.genetics.washington.edu/phylip.html>) with *P. reticulata* as outgroup. For phylogenetic analyses by maximum likelihood the 'Environment for Tree Exploration' (ETE) toolkit <http://etetoolkit.org/> was used. For detection of positive selection, we calculated six different models for null hypothesis or alternative hypothesis. Comparison 1 included two branch specific models: One model with a fixed $\omega = 1$ value (b_neut)

and a second with the marked branch being allowed to evolve independently (b_free). Comparison 2 included two branch-site specific models, model bsA1 (neutral) versus model bsA (positive selection) to identify sites under positive selection on a specific branch. Comparison 3 included two site specific models, model M1 (neutral) and M2 (positive selection). Candidate genes for positive selection were required to be significant in all three comparisons. Genes were considered to be under relaxed selection if ω was significantly different between foreground and background branches (b_free vs. M0) and ω for the foreground branch did not significantly differ from 1 in a comparison between the models b_free and b_neut. For all comparisons a LRT was performed.

Immune genes. Fasta files were searched using the usearch algorithm (<https://www.drive5.com/usearch/>) with user-specific similarity thresholds of 80% for MHC genes and 90% for all innate immunity genes. Genes were assembled using Geneious 8.1.7 (<http://www.geneious.com>) and edited using Bioedit. For the MHC analyses only consensus sequences including an open reading frame of at least 200 bp were included and different MHC alleles were defined using amino acid differences. The innate immunity genes (6 different Toll-like receptors, 1 region of IFN Φ 4, 2 Interleukins, Interferon regulating factor 2, complement components C2 and C3, CLEC, CD59 and TRAF6) were assembled using the *P. formosa* reference gene provided by Ensembl. MEGA was used to calculate genetic distances and phylogenetic tree. Neighbour-joining, maximum likelihood, and minimum evolution produced the same topology in tree reconstruction.

Pairwise relative genetic distance was calculated using the program MEGA v.6.06. For each gene mean values for individuals or species were calculated and compared using ANOVA. ANOVA and Scheffé Posthoc test were done with STATISTICA v.13 (Dell Inc.). Selection analysis (dN-dS; N, nonsynonymous; S, synonymous base exchange) were done using MEGA v.6.06.

Allele-specific gene expression analysis. Six *P. formosa* RNA-Seq sequencing files were downloaded (liver: SRR629501, SRR629518; skin: SRR629511, SRR629503; gills: SRR629508, SRR629510) from SRA and were filtered *P. latipinna* (GCF_001443285.1) and *P. mexicana* (GCF_001443325.1) reference RNA sequences were downloaded from NCBI. To assign ancestral alleles of *P. formosa* genes, sequence homology between ancestral alleles and *P. formosa* genes were identified using Blastn⁷³. When multiple representation of homology was observed, the ancestral allele that generated the longest sequence alignment was kept to represent one ancestral allele of a *P. formosa* gene. Of the 25338 coding genes in the *P. formosa* genome that have a genome feature as 'mRNA', 22118 genes can be assigned to both *P. latipinna* and *P. mexicana* alleles. Short sequencing files generated from *P. formosa* RNA-seq reads were mapped to the ancestral allele reference sequences that are generated by combining sequences of both ancestral alleles using Bowtie2⁷⁴. Customized Perl script was used to retrieve and quantify the short reads that only aligned to one of the ancestral alleles⁷³.

Differential expression between parental alleles was tested using edgeR (<http://bioconductor.org/packages/release/bioc/html/edgeR.html>) (*P. latipinna* alleles were used as control). Log₂(*P. mex* / *P. lat*) was used to label the relativize expression of both ancestral alleles. The false discovery rate (FDR) <0.05 was used to determine whether a gene shows allelic expression bias towards one ancestral allele.

Overall *P. formosa* gene expression was assessed by mapping the RNA-seq sequence reads to the *P. formosa* genome (GCF_000485575.1) using tophat2 (<http://ccb.jhu.edu/software/tophat/index.shtml>), and read counts quantified using featureCounts⁷⁵. A gene was determined to be expressed if at least one sample of the two biological replicates reached a library size-normalized read count (that is, count per million reads) of 0.5.

Detection of segmental duplications, copy number variations. Regions of genomic duplications were estimated across the genomes of 19 *P. formosa* individuals, 5 *P. latipinna* individuals and 4 *P. mexicana* individuals, following an approach based on differences in depth of coverage. We used the RepeatMasker (www.repeatmasker.org) output from NCBI for the *Poecilia_formosa*-5.1.2 assembly and generated output from Tandem Repeat Finder 4.07b, using default parameters. Genomic regions identified by either approach were hard-masked to remove most of the repetitive sequence present in the assembly (Supplementary Table 30). We further sought to identify and mask potential hidden repeats. Scaffolds and contigs were partitioned into 36bps kmers, with adjacent kmers overlapping by 5 bps. These kmers were mapped to the repeat masked version of the assembly using mrsFast 3.3.0, to account for multi-mappings. Over-represented kmers defined as those with more than 42 mappings (accounting for a cumulative proportion of 90% of the mappings) in the assembly (Supplementary Fig. 8) were additionally hard-masked.

We evaluated the overall sequencing performance on the raw reads and demarcated the regions of the reads that displayed the best qualities. We initially mapped the reads to an unmasked version of the assembly using BWA⁶⁰ and removed PCR duplicates using PicardTools (<http://picard.sourceforge.net/>). Non-duplicated reads were then clipped into two consecutive fragments of 36 bps. The resulting reads were then mapped to the prepared kmer masked version of the assembly using mrFast 2.5.0.0. mrCaNaVaR 0.51 was applied to infer the copy number in 1 kb non-overlapping windows of unmasked sequence, that is, the real

window size may exceed 1 kb because it includes any repeat or gap. Notably, as reads will not map to the genomic coordinates masked in the assembly, a spurious drop off in read depth estimates might appear at the edges of masked regions, which could underestimate the copy number inferences in later steps. To prevent this, the 36 bps flanking any masked region or gap were also masked and thus not included in the window definition. Genome-wide read depth distribution was calculated by iteratively excluding windows with extreme read depth values relative to the normal distribution and the remaining windows defined as control regions. The mean read depth in these control regions was considered to correspond to a copy number equal to two and used to convert the read depth value in each window into a GC-corrected absolute copy number (see Supplementary Figs. 9 and 10 for distribution in copy number values in control regions and per sample duplication content for all *P. formosa* samples, Supplementary Figs. 11 and 12 for the parental *P. latipinna* and *P. mexicana* samples).

We defined segmental duplications (SDs) as at least five consecutive windows of non-overlapping non-masked sequences with copy number values higher than the mean plus three standard deviations, allowing one of the windows to have a copy number value that is larger than the mean plus two standard deviations. Cutoffs were defined on a per sample basis. Furthermore, regions with an absolute copy number above 100 in any sample were excluded. Gaps were removed from the called intervals in downstream analysis.

GO enrichment analyses on the set of duplicated genes were performed using R and the topGO package on a per species basis. First, the Ensembl IDs of the duplicates genes were mapped to the corresponding GO terms using the biomaRT package. GO enrichment was studied for the GO ontologies 'Biological Process' and 'Molecular Function'. We used Fishers exact test to detect enrichments, and corrected the raw *P* values with the Benjamini-Hochberg method.

Paternal introgression. To determine whether paternal introgression events had occurred in any of the analysed samples, we assessed the existence of allele imbalance at heterozygous sites across all scaffolds larger than 100 Kb. Sequencing reads were aligned to the *P. formosa* reference assembly using BWA⁶⁰ v.0.7.4 with default parameters and PCR duplicates were removed using PicardTools (<http://picard.sourceforge.net/>). SNPs were called using FreeBayes (v.0.9.14) with the following parameters: standard-filters, no-population-priors, report-genotype-likelihood-max. We retained for subsequent analysis mapped, biallelic heterozygous SNPs with a minimum QUAL >= 30 and a minimum DP >= 5. We also required at least 20% of the reads supporting each SNP derived from the minor allele. In the absence of introgression allele frequencies should be approximately 50% of the sites. In the case of paternal introgression a distorted frequency is expected skewed towards alleles that are present in the paternal species. We identified putative paternal introgression events as bimodal distributions of the reference allele frequency that result from the presence of an extra copy inherited from the male host. We confirmed that the inferred copy number throughout the identified scaffolds was higher in the samples where the paternal introgression was detected. To trace back the imbalanced allele to the corresponding paternal species, sequencing data derived from five *P. mexicana* and five *P. latipinna* fishes was used to call SNPs using the approach described above. After filtering, fixed homozygous sites with opposing genotypes in the two species were identified by the following criteria: either all *P. mexicana* samples were homozygous for the reference allele and all *P. latipinna* samples were homozygous for the alternative allele, or the other way around. These fixed homozygous sites were then intersected with heterozygous variants called in each of the 19 *P. formosa* samples and for each site the percentage of reads carrying the allele present in *P. mexicana* was calculated. In the absence of paternal introgression, this frequency should be ~50%. If there is an introgressed scaffold, the imbalanced allele at heterozygous sites should match the allele present in the corresponding paternal species, and thus this frequency would deviate from 50%.

To model the relief from genomic decay allowed by paternal introgression the equations described in a previous study¹² were used with parameter estimates changed as stated.

Life Science Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. All assemblies are available at Genbank under the following accession numbers: GCF_000485575.1 (*Poecilia_formosa*-5.1.2) BioProject PRJNA89109, GCA_001443325.1 (*Poecilia_mexicana*-1.0) BioProject PRJNA196869, and GCA_001443285.1 (*Poecilia_latipinna*-1.0), BioProject PRJNA196862. Accession numbers for population sample genome reads are given in Supplementary Table 3.

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Author contributions

W.C.W. and M.S. initiated and managed the genome project. P.M., C.T., and L.H. built the assembly, B.A., D.N.M. generated the Ensembl gene annotation, K.P. led the NCBI gene annotation, F.F., M.M. for annotation of gene variants, G.W.C.T. and M.W.H. did the gene family analysis, D.C. and J.N.V. performed the repeat and TE analyses, M.St., K.L., and J.W. performed the mtDNA analyses, K.L. performed the immune gene analyses, S.K., Z.W., and M.S. performed the analysis of male specific, TE silencing machinery and meiosis genes, S.X., M.L. performed and reviewed the analysis of heterozygosity and gene conversion, C.M.G. participated in the population analyses, collection and sample identification, R.G.P., L.F.K. and T.M.B. did the introgression and segmental duplication analysis, L.L. did the modeling of genomic decay, Y.L. and R.B.W. performed the ASE analyses, S.K. and M.S. performed the selection analysis, all authors contributed to data interpretation, W.C.W. and M.S. wrote the manuscript.

Competing interests

The authors declare no competing financial interests.

Additional information

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▶ Experimental design

1. Sample size

Describe how sample size was determined.

In our study no quantitative experiments were performed which compared different groups

2. Data exclusions

Describe any data exclusions.

No data were excluded

3. Replication

Describe whether the experimental findings were reliably reproduced.

All attempts at replication were successful

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

See 1

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

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6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
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7. Software

Describe the software used to analyze the data in this study.

All software used is publicly available and clearly described in the methods section or main text of the manuscript

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► Materials and reagents

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8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cell lines were used

b. Describe the method of cell line authentication used.

No eukaryotic cell lines were used

c. Report whether the cell lines were tested for mycoplasma contamination.

No eukaryotic cell lines were used

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

No commonly misidentified cell lines were used

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Pfo_Bar-1 female, big river, high population density, Rio Purification near Barretal P. mexicana N 24°02.848'
W 98° 22.264'

Pfo_Tan-1 female, sulfur creek, Taninul, Sulfur Creek P. mexicana N 21°56.363'
W 98° 53.296'

Pfo_Tan-2 female, sulfur creek, Taninul, Sulfur Creek P. mexicana N 21°56.363'
W 98° 53.296'

Pfo_Tan-3 female, sulfur creek, Taninul, Sulfur Creek P. mexicana N 21°56.363'
W 98° 53.296'

Pfo_Tan-4 female, sulfur creek, Taninul, Sulfur Creek P. mexicana N 21°56.363'
W 98° 53.296'

Pfo_Vic-1 female, small ditch, Highway 80, km 105 north of Ciudad Mante P. mexicana, P. latipunctata N 22° 48' 44.280"
W 99° 0' 45.000"

Pfo_Cha lake, female, still water, coastal area, polluted, medium population density Laguna Champaxan P. mexicana, P. latipinna N 22°23.425'
W 97° 55.828'

Pfo_Gua female, fast flowing small river, low population density Rio Guayalejo P. mexicana N 23°16.624'
W 98° 56.315'

Pfo_Bar-2 female, big river, high population density, Rio Purification near Barretal P. mexicana N 24°02.848'
W 98° 22.264'

Pfo_D1 female, small ditch Ditch1 P. latipinna N 25° 59' 11.400"
W 97° 31' 52.680"

Pfo_Olm female, Olmito P. latipinna N 25° 59' 9.240"
W 97° 31' 53.760"

Pfo_Bar-3 female, big river, high population density Rio Purification near Barretal P. mexicana N 24° 02.848'
W 98° 22.264'

Pfo_Br female, big river, high population density, near Brownsville P. latipinna N 25°
52' 58.812"

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants