

SPECIAL ISSUE: THE MOLECULAR MECHANISMS OF ADAPTATION AND SPECIATION: INTEGRATING GENOMIC AND MOLECULAR APPROACHES

Demographic modelling with whole-genome data reveals parallel origin of similar *Pundamilia* cichlid species after hybridization

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Abstract

Modes and mechanisms of speciation are best studied in young species pairs. In older taxa, it is increasingly difficult to distinguish what happened during speciation from what happened after speciation. Lake Victoria cichlids in the genus *Pundamilia* encompass a complex of young species and polymorphic populations. One *Pundamilia* species pair, *P. pundamilia* and *P. nyererei*, is particularly well suited to study speciation because sympatric population pairs occur with different levels of phenotypic differentiation and reproductive isolation at different rocky islands within the lake. Genetic distances between allopatric island populations of the same nominal species often exceed those between the sympatric species. It thus remained unresolved whether speciation into *P. nyererei* and *P. pundamilia* occurred once, followed by geographical range expansion and interspecific gene flow in local sympatry, or if the species pair arose repeatedly by parallel speciation. Here, we use genomic data and demographic modelling to test these alternative evolutionary scenarios. We demonstrate that gene flow plays a strong role in shaping the observed patterns of genetic similarity, including both gene flow between sympatric species and gene flow between allopatric populations, as well as recent and early gene flow. The best supported model for the origin of *P. pundamilia* and *P. nyererei* population pairs at two different islands is one where speciation happened twice, whereby the second speciation event follows shortly after introgression from an allopatric *P. nyererei* population that arose earlier. Our findings support the hypothesis that very similar species may arise repeatedly, potentially facilitated by introgressed genetic variation.

Keywords: adaptive radiation, cichlid fish, demographic modelling, ecological speciation, hybrid speciation

Received 15 March 2016; revision received 15 August 2016; accepted 22 August 2016

Introduction

Young species pairs are especially valuable in speciation research because differences between them can be

attributed with some confidence to processes operating during speciation. In older species with complete reproductive isolation, it is difficult to distinguish factors that contributed to reproductive isolation and divergence during speciation from those that only occurred after speciation (Coyne & Orr 2004). However, most young

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sister species pairs have emerged from singular evolutionary events and hence provide only one snapshot in the presumed continuum between a single panmictic population and two reproductively isolated species (Nosil & Feder 2013; Faria *et al.* 2014; Seehausen *et al.* 2014).

Natural replicates, such as similar species pairs that have evolved under similar contrasting selection regimes (Schluter & Nagel 1995), allow for powerful tests of the underlying processes leading to speciation, and the role of natural and sexual selection in speciation (Schluter & Nagel 1995; Jones *et al.* 2012; Nosil 2012; Butlin *et al.* 2014). Consistent phenotype–environment correlations suggest the repeated action of natural selection in shaping trait values, and traits or loci that differ consistently between species in replicated species pairs are strong candidates for divergent selection and/or involvement in reproductive isolation (Schluter & Nagel 1995; Rundle *et al.* 2000; Schluter *et al.* 2004; Faria *et al.* 2014). In many studies of repeated evolution, the genetic basis of traits underlying similar phenotypes is unknown. Nevertheless, the traits that evolved repeatedly in parallel are often assumed to have arisen independently through separate *de novo* mutations (narrow-sense definition of parallel evolution), but such mutations could also have been recruited from shared ancestral polymorphisms or interspecific gene flow (Schluter & Conte 2009; Johannesson *et al.* 2010; Faria *et al.* 2014). The distinction between ‘parallel evolution’ and ‘convergent evolution’ (independent evolution of similar phenotypes in distantly related lineages or based on different pathways) is disputed (Haas & Simpson 1946; Arendt & Reznick 2008; Conte *et al.* 2012). Here, we use the term parallel evolution as our study species are very closely related. A special form of parallel evolution, parallel speciation, is when reproductive isolation (RI) in several speciation events also evolves in parallel, driven by the same mechanism. In this case, species are reproductively isolated from their sister species, but would potentially lack RI against independently evolved, geographically distant species experiencing similar selection environments and expressing similar phenotypes (Schluter & Nagel 1995). Parallel evolution of reproductive isolation based on the same traits may occur through *de novo* mutations, parallel sorting of standing genetic variation or introgressed alleles. In all of these cases, there are independent replicates of the speciation event, even though the alleles involved may have arisen only once and may be shared between the speciation events.

Another type of evolutionary replication, applying not to the origin of species but to the maintenance of species differences, is when a young species pair hybridizes at several independently established secondary

contact zones (or geographically isolated instances of breakdown of reproductive isolation between species in primary contact, e.g., due to habitat disturbance), and shows parallel maintenance of species differences despite gene flow (Barton & Hewitt 1985; Rand & Harrison 1989; Abbott *et al.* 2013). Genetic regions that show reduced introgression in independent hybrid zones likely contain loci that are under divergent selection, or contribute to reproductive isolation between the species (Barton & Hewitt 1989; Rieseberg *et al.* 1999; Rieseberg & Buerkle 2002; Gompert & Buerkle 2009; Teeter *et al.* 2010; Harrison & Larson 2016). Studying such situations allows investigation of the genomic architecture of adaptation and reproductive isolation, the types of incompatibilities and the roles of different types of selection. If a species pair is young, then the mechanisms that can be inferred are likely to have played a role in initiating speciation too, whereas this inference cannot be made when hybrid zones between older species are being studied (Nosil & Schluter 2011).

Systems with either parallel speciation or parallel maintenance of species differences in multiple hybrid zones are thus both useful for studying the processes underlying speciation. However, distinguishing between these scenarios is not trivial as they can both produce stronger neutral genetic clustering by geographical proximity than by phenotypic similarity (Johannesson *et al.* 2010; Bierne *et al.* 2013; Faria *et al.* 2014). It is crucial to know the evolutionary history of these natural replicates to assess the level of evolutionary independence and to study the underlying evolutionary mechanisms, including natural and sexual selection, *de novo* mutations, gene flow, drift and standing genetic variation. Importantly, parallel speciation has been taken to imply that speciation is to some extent a predictable outcome of adaptation, whereas parallel maintenance of species differences does not allow this inference (Stern & Orgogozo 2009; Rosenblum *et al.* 2014). Genetic clustering of species and ecotypes by geographical proximity rather than by phenotypic similarity has been shown in species complexes of sunflowers (Renaut *et al.* 2014), sticklebacks (Rundle *et al.* 2000), *Littorina* periwinkle snails (Butlin *et al.* 2014; Ravinet *et al.* 2015), Darwin finches (Lamichaney *et al.* 2015), *Timema* stick insects (Nosil *et al.* 2002) and cichlid fishes of Lake Malawi (Allender *et al.* 2003) and Victoria (Seehausen *et al.* 2008; Konijnendijk *et al.* 2011; Magalhaes *et al.* 2012). However, tests discriminating single and multiple origins of ecotypes or species in the face of continuing gene flow are often lacking (but see Butlin *et al.* 2014). Here, we explicitly test alternative scenarios for speciation in *Pundamilia* cichlids using genomic data and demographic modelling.

Lake Victoria haplochromine cichlids are an adaptive radiation of approximately 500 endemic species that evolved over the past 15 000 years (Seehausen 2006). They comprise multiple replicates of young species pairs differing along the same dominant axes of phenotypic and ecological divergence, most strikingly in male nuptial coloration and water depth occupation, whereas less closely related species can also differ dramatically in morphology and ecology (Seehausen 2015). The Lake Victoria endemic genus *Pundamilia* offers a unique opportunity to study speciation in natural replicates with varying extents of reproductive isolation, of what appears to be the same sympatric species pair at different sites within the lake. The sister species *Pundamilia pundamilia* and *P. nyererei* coexist in complete sympatry at many sites in Lake Victoria (Fig. 1), including islands in the Mwanza Gulf, an environment with considerable variation in water turbidity between sites. At islands with clear water, *P. pundamilia* and *P. nyererei* are distinct species with highly divergent male nuptial coloration, some differences in ecology and morphology, and strong assortative mating (Fig. 1c, Table S1, Supporting information, Seehausen 2009).

Pundamilia cichlids are a model system for studying the interaction of sexual and natural selection in speciation and the role of sensory drive mechanisms, as visual system adaptation to contrasting light environments

between the sympatric sister species is associated with divergent sexual selection on male nuptial coloration (Maan *et al.* 2006; Seehausen *et al.* 2008). In Lake Victoria, particulate matter selectively absorbs and scatters short-wavelength light (Okullo *et al.* 2007) shifting the light gradient from broad-spectrum daylight in shallow water towards more red light in deeper water. This light gradient is steeper in more turbid water and less steep in clear waters (Seehausen *et al.* 1997). *P. nyererei*, which lives in deeper water with a relatively more red-shifted light spectrum, has bright red male nuptial coloration and visual sensitivity shifted towards red light relative to *P. pundamilia* (Table S1, Supporting information, Maan *et al.* 2006). In addition, *P. nyererei* females, cryptically yellow-brown themselves, choose their mates based on nuptial coloration, preferring conspecific red over heterospecific blue males (Seehausen *et al.* 1997; Seehausen & van Alphen 1998; Selz *et al.* 2014b) and have a preference for redder males (Maan *et al.* 2004, 2010; Stelkens *et al.* 2008; Selz *et al.* 2016). In contrast, the more shallow breeding *P. pundamilia* has blue male nuptial coloration (Seehausen 1996), visual sensitivity that is more shifted towards blue light (Maan *et al.* 2006), and females prefer conspecific blue over heterospecific red males (Table S1, Supporting information, Seehausen *et al.* 1997; Seehausen & van Alphen 1998; Selz *et al.* 2014b). The two species also differ in

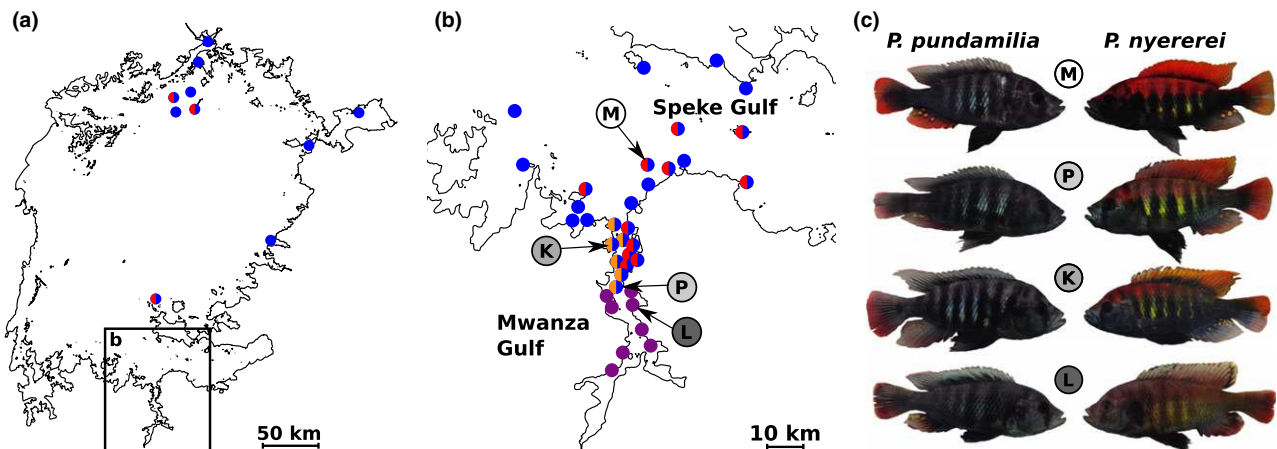


Fig. 1 Distribution and sampling sites of *Pundamilia pundamilia* and *P. nyererei*. (a) Map of Lake Victoria showing the known records of *P. pundamilia* (blue), *P. nyererei* (red) and sites harbouring both species (half red, half blue circles). The square indicates the location of the Mwanza Gulf and Speke Gulf enlarged in (b). The two types of *P. nyererei*, deep-bodied with blueish belly and slender-bodied without blueish belly (see Table S1), are marked with orange and dark red, respectively. Sites in the turbid South of the Mwanza Gulf inhabited by colour-polymorphic *Pundamilia* populations which are not clearly identifiable to a species are shown with purple circles. Sampling sites of fish used in this study are indicated with single letter abbreviations [Makobe Island (M), Kissenda Island (K), Python Island (P), Luanso Island (L)]. The darkness of the grey background in the symbols reflects the water turbidity (darker means more turbid). Note that although Kissenda Island is closer to the open lake than Python Island, the former is more turbid as it lies inside a bay. Distribution records are assembled from Seehausen (1996, 1997), Seehausen *et al.* (1998) and Seehausen (2009). (c) Representative individuals of blue and red *Pundamilia* found at the different sampling sites. At Makobe, Kissenda and Python Islands, they represent the nominal species of *P. pundamilia* and *P. nyererei*, whereas for Luanso Island, red and blue individuals were selected from the polymorphic population.

many other traits such as female coloration, and multiple morphological and ecological characteristics (Table S1, Supporting information).

In this study, we focus on species pairs from islands with different water turbidity and hence different steepness of the ambient light gradient. Higher turbidity and steeper light gradients are associated with weaker genetic differentiation between *P. pundamilia* and *P. nyererei* (Seehausen *et al.* 2008). Whereas the species are clearly distinct at the clear water Makobe Island in the open lake just north of the Mwanza Gulf, hybrids can be seen regularly at the intermediately turbid sites of Kissenda Island and Python Island in the Mwanza Gulf (Fig. 1). Further south in the Gulf, at Luanso Island, the water is even more turbid and *Pundamilia* males vary extensively in nuptial coloration, including clearly red and clearly blue individuals, but intermediate phenotypes are most abundant and genetic data are consistent with a single panmictic population (Seehausen *et al.* 2008; van der Sluijs *et al.* 2008). Likewise, female preference for male coloration has a bimodal frequency distribution at the islands where male coloration is bimodally distributed, but has a unimodal frequency distribution at Luanso Island where most females show no behavioural mating preference and few females show preferences for either red or blue males (Seehausen *et al.* 2008; van der Sluijs *et al.* 2008).

Populations of *P. nyererei* exhibit considerable phenotypic variation both in morphology and in chroma (*i.e.* purity and intensity of colour) that can be classified into two types (Seehausen 1996). One type occurs mainly in the western Mwanza Gulf (e.g. Python and Kissenda Islands), whereas the other type occurs in the eastern Mwanza Gulf and in the main body of Lake Victoria (e.g. Makobe Island, Fig. 1, Seehausen 1996). They differ mainly in body depth, jaw length, and female and male coloration (Fig. 1c, Table S1, Supporting information, Seehausen 1996). *P. nyererei* occupies the entire depth range at Kissenda and Python Islands, whereas at Makobe Island it lives almost exclusively deeper than 3 m (Seehausen 1997; Seehausen *et al.* 2008). The *P. pundamilia* populations at different islands used in our study are similar in coloration (Castillo Cajas *et al.* 2012), but they differ in relative head length, eye size and in the number and shape of the vertical bars (Table S1, Supporting information, Seehausen 1996; Seehausen *et al.* 1998). These population differences may have arisen through independent evolution after speciation and range expansion, or they may suggest that populations allocated to the same nominal species evolved more than once in parallel.

Seehausen *et al.* (2008) found that neutral genetic divergence (based on microsatellite data) between sympatric *P. pundamilia* and *P. nyererei* within an island was

often lower than that between allopatric populations belonging to the same morphologically defined nominal species. This observation raised the question of whether interspecific gene flow in sympatry had eroded the phylogenetic signature of common ancestry of all *P. nyererei*, or whether similar red and blue *Pundamilia* species pairs had evolved in parallel at different islands. Whereas *P. pundamilia* (blue) is found at almost every sampled rocky habitat patch in Lake Victoria (Seehausen *et al.* 1998) and has the highest record of sympatry with other *Pundamilia* species (Seehausen 1996; Seehausen & van Alphen 1999), the distribution range of *P. nyererei* (red) is patchy, and *P. nyererei* populations are only found at sites where *P. pundamilia* is also present (Seehausen & van Alphen 1999). It has thus been proposed that *P. pundamilia*-type fish (blue males) may represent the ancestral form that colonized the different islands and rocky mainland shores of Lake Victoria (Seehausen 1996, 1997; Seehausen & Schluter 2004; Dijkstra *et al.* 2007). Populations of *P. nyererei* (red males) occur only at some islands, with a patchy geographical distribution, and may have evolved independently at different islands or, alternatively, *P. nyererei* may have split once from *P. pundamilia* and subsequently colonized the different islands where it experienced gene flow with the resident *P. pundamilia* populations (Seehausen 1996, 1997; Seehausen & Schluter 2004; Dijkstra *et al.* 2007).

Distinguishing these evolutionary scenarios has important consequences for understanding the speciation process and the effects of environmental variation on speciation and persistence of sister species. Here, we test alternative evolutionary scenarios including, single, parallel and hybrid origin models using whole-genome and partial genome sequences of population samples, and demographic modelling.

Materials and methods

Samples

Wild males of *P. nyererei* and *P. pundamilia* were collected with gill nets and by angling at Makobe Island and at three islands in the Mwanza Gulf (Kissenda, Python and Luanso Islands, Fig. 1) in 2005 and in 2010. Phenotypically intermediate males were also sampled at islands where they occurred (see Table S2, Supporting information for more sample information). DNA was extracted from fin clips using a standard phenol-chloroform protocol (Sambrook & Russell 2001).

RAD sequencing

Restriction-site associated DNA sequencing (RADseq) was performed following a standard protocol (Baird

et al. 2008) with minor modifications. Restriction digestion was performed overnight using the restriction endonuclease HF-*Sbf*I (NewEngland Biolabs) and 1 µg DNA per sample. P1 adapters contained 5- to 8-bp-long barcodes differing by at least two nucleotides from all other barcodes. The DNA was sheared with a Covaris S220 Focused-Ultra sonicator and fragments of 300–600 bp length were manually cut from an agarose gel. All libraries were single-end-sequenced on an Illumina HiSeq 2500 sequencer. The reads were demultiplexed and trimmed to 84 bp with the `process_radtags` script from the Stacks pipeline (Catchen *et al.* 2013), correcting single errors in the barcode and the restriction site, and discarding reads with incomplete restriction sites. The FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit) was used to remove all reads containing at least one base with a Phred quality score below 10 and reads with more than 10% of bases with quality less than 30. The reads of each individual were then mapped to the *P. nyererei* reference genome (Brawand *et al.* 2014) using Bowtie2 v. 2.2.6 (Langmead & Salzberg 2012) with the end-to-end alignment option and default parameters. Base score recalibration was performed using empirical error rate estimations derived from bacteriophage PhiX reads sequenced together with the RAD libraries following Marques *et al.* (2016). Single nucleotide polymorphisms (SNPs) and genotypes were called using GATK Unified Genotyper v. 3.5 (McKenna *et al.* 2010). All sites were then filtered with a custom-made Python script, BCFTOOLS v. 0.1.12, and VCFTOOLS v. 0.1.14 (Danecek *et al.* 2011). We removed sites within 5 bp from indels (insertions/deletions) to avoid false SNPs due to potential misalignment problems. Genotypes were required to have a depth of coverage of at least 10 reads and a minimum quality value (GQ) of 30. Sites with more than 25% missing data were removed. Sites with a mean genotype depth greater than $59.6 \times$ (1.5 times the interquartile range from the mean) were excluded as they are expected to be enriched for paralogs. Indeed, 25% of the SNPs with minor allele frequency of at least 0.05 among these sites were heterozygous in over 80% of the individuals.

Genetic distances

To study population structure among our populations, we performed principal component analysis (PCA) with the R-package SNPRelate (Zheng *et al.* 2012) using biallelic SNPs from the RAD sequencing data set with a minor allele frequency of at least 5% over all sequenced individuals. A data set of unlinked SNPs was obtained by pruning SNPs that were in high linkage disequilibrium (LD) across all populations combined. We used `plink` ('-indep-pairwise 50 5 0.5', Purcell *et al.* 2007) to

calculate LD (r^2) between each pair of SNPs in a window of 50 SNPs. If a pair of SNPs had r^2 greater than 0.5, one of the two SNPs was removed at random. The window was then shifted by five SNPs and the procedure was repeated until the last SNP was reached.

To measure genetic distances between the populations, we created a RAD data set without missing data by randomly sampling 12 genotypes of each population per site. Individuals phenotypically identified as putative hybrids were excluded. We used all biallelic sites in which the rarer allele was observed at least three times across all individuals (minor allele frequency 1.5%) to calculate pairwise F_{ST} values. We applied a Mantel test with 1000 permutations to assess whether genetic distances are correlated with waterway distances between the islands using ARLEQUIN v. 3.5.2.3 (Excoffier & Lischer 2010).

Whole-genome sequencing

Whole-genome sequencing data were generated for a subset of the same individuals (four individuals of *P. pundamilia* and four of *P. nyererei* each from Makobe and Python Islands, see Table S2, Supporting information) using PCR-free library preparation (Kozarewa *et al.* 2009) and Illumina HiSeq 3000 paired-end sequencing. To avoid any sequencing lane effects and to get an even read representation, all individuals were given separate barcodes and subsequently sequenced together on four Illumina HiSeq 3000 lanes. Local alignment against the *P. nyererei* reference genome (Brawand *et al.* 2014) was performed with Bowtie 2 v. 2.2.6 (Langmead & Salzberg 2012), and variant calling and genotyping with Haplotype Caller (GATK v. 3.5, McKenna *et al.* 2010). To avoid potential paralogous regions, we excluded sites with a mean genotype depth greater than 1.5 times the interquartile range from the mean and we excluded sites deviating from Hardy–Weinberg equilibrium (HWE) due to excess observed heterozygosity over all 16 individuals pooled together using VCFTOOLS v. 0.1.14 (Danecek *et al.* 2011) and a *P*-value cut-off of 0.001. We did not have enough power to filter for deviation from HWE in single populations. All sites were required to have a depth of coverage of at least 15 reads in each individual. All biallelic SNPs were then extracted for demographic modelling.

To assess whether the individuals used for whole-genome sequencing differed in ancestry proportions from the other individuals of their respective populations in the RAD data set, we performed Bayesian clustering assignment of all individuals with two to seven clusters ($K = 2-7$) using STRUCTURE v. 2.34 (Pritchard *et al.* 2000). We used all biallelic sites with a minor allele frequency of 5% in the RAD data set. We ran 10

replicates each assuming two to seven clusters with 500 000 burnin and 1 000 000 sampling steps. The most likely number of clusters was identified by the highest delta K among all runs (Evanno *et al.* 2005) with STRUCTURE HARVESTER (Earl & vonHoldt 2012). We used the greedy algorithm within CLUMPP v. 1.1.2 (Jakobsson & Rosenberg 2007) to summarize across variation in the 10 replicates for each K.

Demographic modelling

To discriminate among alternative evolutionary scenarios for the origin of the *P. nyererei* and *P. pundamilia* populations and to estimate relevant parameters, we used demographic modelling based on the minor allele site frequency spectrum (SFS) of the whole-genome sequencing data. First, we used only the populations at Makobe Island, where the two species are most distinct, to assess whether speciation occurred in a period of geographical isolation or in the face of gene flow (Fig. 3). Next, we contrasted alternative demographic models for the evolution of the species pairs at Makobe (representing islands in the open lake) and Python (representing islands in the western Mwanza Gulf) combined. We compared models of single, parallel and hybrid origin of the red and the blue species, each combined with different post-speciation gene flow scenarios (Fig. 4).

For each model, the fit to the observed multidimensional minor allele SFS was maximized using the composite-likelihood method implemented in FASTSIMCOAL v. 2.5.2 (Excoffier *et al.* 2013) with the following options: -N 100 000 (number of coalescent simulations), -L 40 [number of expectation-maximization (EM) cycles], -M 0.001 (minimum relative difference in parameter values for the stopping criterion) and -C 10 (threshold for observed SFS entry count, pooling all entries with less than 10 SNPs). For all model parameters, we used wide search ranges with log-uniform distributions (see Tables S3 and S4, Supporting information). For each demographic model, we performed 500 independent fastsimcoal 2 runs to determine the parameter estimates leading to the maximum likelihood (Excoffier *et al.* 2013).

The observed multidimensional SFS was computed with ARLEQUIN v. 3.5.2.3 (Excoffier & Lischer 2010). As we currently lack a good estimate for the mutation rate in Lake Victoria cichlids, all parameter estimates are relative to the time of the first split in each model, which was fixed to be 6000 generations before present. Although the real splitting time between *P. pundamilia* and *P. nyererei* is likely more recent, this initial splitting time provides an upper bound, as it roughly coincides with the refilling of the Lake Victoria basin after several

thousand years of complete desiccation (Johnson *et al.* 2000), assuming a generation time of two years. Note that the species pair and the entire genus *Pundamilia* are endemic to Lake Victoria itself and are absent from the other smaller lakes in the region. The species can only have arisen after the lake filled again with water. Many polymorphisms will not have arisen in the past 6000 generations but rather represent ancestral standing variation. In the demographic models, the levels of ancestral standing variation are represented by the effective size of the ancestral population.

The best fitting demographic model was identified on the basis of the Akaike's information criterion (AIC, Akaike 1974). However, we note that given the presence of linked sites in our data, the reported AIC values should be interpreted with caution (Excoffier *et al.* 2013). For this reason, we also examined the likelihood distributions obtained based on 100 expected SFS, each approximated using 1 million coalescent simulations under the parameters that maximize the likelihood for each model. These distributions inform us about the variance in likelihoods estimated by fastsimcoal2. An overlap of these distributions between models would indicate no significant difference between the fit of alternative models as the difference may be attributed to the variance in the SFS approximation.

To get confidence intervals for the parameter estimates, we used a nonparametric block-bootstrap approach. To account for LD, 200 bootstrap data sets were obtained by dividing the SNPs into 100 blocks, and sampling with replacement 100 blocks for each bootstrap data set, to match the original data set size. The parameter point estimates from the run with the highest likelihood (of 100 independent runs) from each bootstrapping replicate were then used to compute 95 percentile confidence intervals with the R-package 'boot' (Canty & Ripley 2015).

Results

Data sets

The total width of the data matrix derived from RAD sequencing, after alignment to the reference genome and genotype calling, was 3 102 689 bp (including invariant sites). After all filtering steps, we retained 21 090 SNPs with maximum 25% missing data at a minor allele frequency level of 0.01. The mean proportion of missing data in the filtered data set was 8.7% in the whole RADseq data set, and the mean depth of coverage was 40 \times .

The whole-genome sequencing data set after mapping to the *P. nyererei* reference genome contained a total of 653 million bp with a minimum depth of coverage of

5 \times and a maximum of 50% missing data. The mean missing data proportion was 1.5%, and average depth of coverage was 19 \times . The data set used for the demographic modelling contained 433 893 biallelic SNPs without missing data and a minimum depth of coverage of 15 \times in all 16 individuals.

Population structure

The RADseq data set confirms previous finding by Seehausen *et al.* (2008) that *P. nyererei* and *P. pundamilia* populations coexisting at the same island tend to be genetically more similar to each other than are allopatric populations of the same nominal species from different islands (Figs 2 and S1, Supporting information). Interspecific F_{ST} values are highest at the Makobe clear water site, intermediate at Kissenda and Python and zero at Luanso Island, the most turbid site (Figs 2a and S1, Supporting information). Intraspecific F_{ST} values among island populations of *P. pundamilia* correlate positively with geographical distance (Mantel test, $P = 0.048$), whereas the correlation is nonsignificant for *P. nyererei* ($P = 0.176$), fully consistent with earlier microsatellite-based inference (Seehausen *et al.* 2008).

In the PCA, the first component (PC1) carries a signature of geographical distance between islands but also

differentiates the sympatric species within islands. PC2 differentiates the sympatric species, but the species diverge in opposite directions at Makobe versus at Kissenda and Python Islands. The third principal component groups populations by nominal species (Fig. 2b). Individuals from Luanso Island, thought to be a population of intermediates or natural hybrids, form a single cluster, intermediate between the *P. pundamilia* and *P. nyererei* regions in PCA space, without evidence of colour-associated genetic differentiation. On PC1, Luanso individuals form a distinct cluster clearly separated from all other islands. At higher PC axes, all Luanso individuals form a cluster in the centre of the PCA space. Several individuals from Python and Kissenda that were phenotypically identified as putative hybrids are located between the clusters of *P. nyererei* and *P. pundamilia* populations of their respective islands.

Demographic modelling

To test whether *P. nyererei* and *P. pundamilia* speciated in allopatry (*i.e.* without gene flow) or in the face of gene flow, and whether gene flow changed over time, we contrasted different scenarios of speciation with gene flow and without gene flow using the Makobe

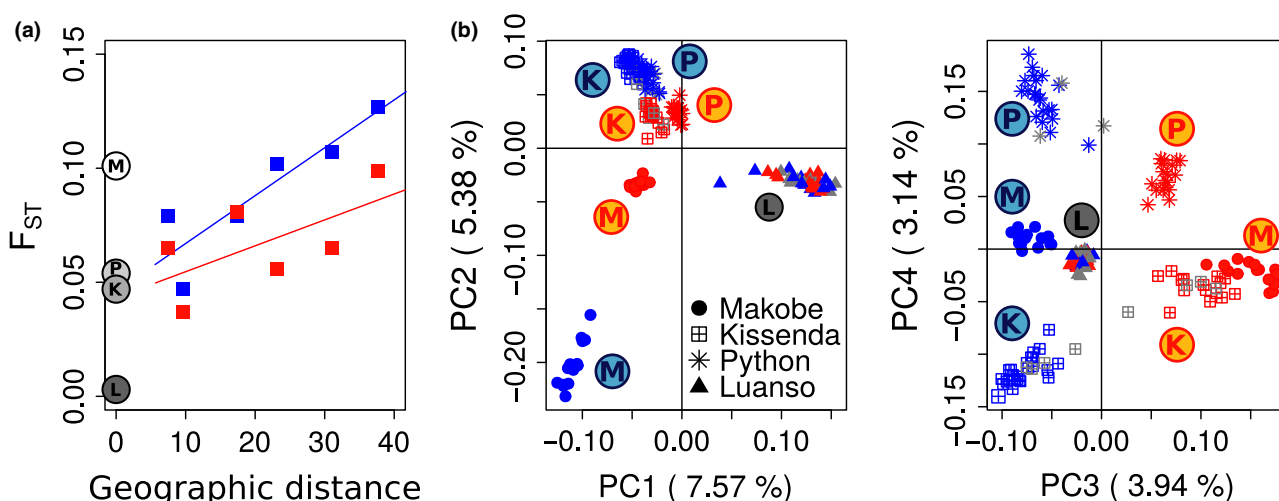


Fig. 2 The major axis of genetic diversity does not separate the nominal species but shows an isolation-by-distance pattern. (a) Correlation of geographical distance with pairwise F_{ST} values between red populations (*nyererei*-like, red squares, not significant) and between blue populations (*pundamilia*-like, blue squares, Mantel test P -value = 0.048) from different islands [Makobe (M), Python (P), Kissenda (K) and Luanso (L)]. Interspecific F_{ST} values between species in sympatry are shown as grey circles for each island at zero geographical distance. As the *Pundamilia* at Luanso Island cannot be clearly assigned to a nominal species, red and blue individuals were identified by eye and used to estimate F_{ST} . The estimates are based on 15 017 biallelic sites (for pairwise F_{ST} comparisons between all populations, see Fig. S1, Supporting information). (b) PCA showing the genetic similarity between the sampled individuals based on 13 213 biallelic sites from the RADseq data set. The first four axes are shown with the percentage of variance explained in parentheses. Different symbols represent individuals from different sampling sites as indicated in the legend. For Makobe, Python and Kissenda Islands, the red, blue or grey colour indicates that an individual is phenotypically identified as *P. nyererei*, *P. pundamilia*, or as a potential hybrid, respectively. The Luanso Island individuals with most red or least red are shown with red or blue triangles, respectively, and others with grey triangles.

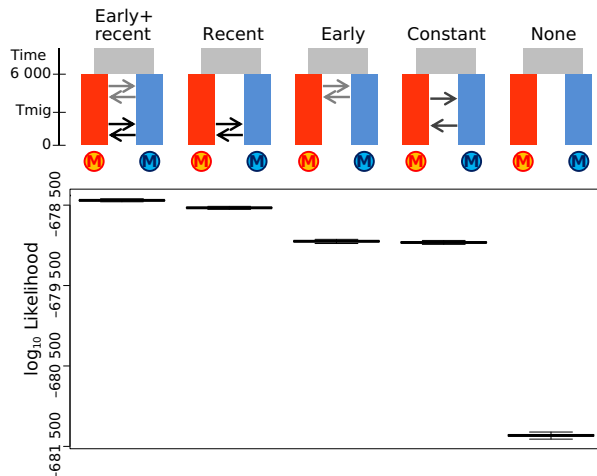


Fig. 3 Comparing alternative gene flow scenarios reveals that a model of speciation with two different migration periods fits the data best. Tested models of the evolution of *P. nyererei* (red) and *P. pundamilia* (blue) at Makobe Island ordered by likelihood with boxplots showing the \log_{10} likelihood distributions inferred by recalculating the \log_{10} likelihoods for 100 simulated SFS. The first model (early + recent) allows for two periods with different migration rates (different early and recent gene flow matrices). The time of migration matrix change is inferred as a model parameter (T_{mig}). The second best model (recent) allows only for gene flow starting at T_{mig} until the present. The early gene flow model allows only for gene flow up to time T_{mig} , and the constant gene flow model simulates ongoing migration that remains constant since the speciation time. The last model does not allow for gene flow.

populations, because these were least affected by recent changes in water clarity. We compared five models with (1) secondary contact (only recent gene flow), (2) only early gene flow, (3) different amounts of early and recent gene flow, (4) constant gene flow and (5) no gene flow (Fig. 3). The time of change in gene flow (*i.e.* start of recent or end of early gene flow) was estimated as a model parameter and allowed to range from 1 to 6000 generations. Parameter estimates and likelihoods are given in Table S3 (Supporting information).

The highest likelihood was obtained for a model of speciation with gene flow, in which the rate of gene flow has recently increased (Fig. 3, Table S3, Supporting information). The change in the rate of gene flow was inferred to have occurred about 420 generations (~840 years) ago. However, these time estimates should be interpreted with caution as we fixed the first split to the upper bound of 6000 generations ago (corresponding roughly to the time since refilling of Lake Victoria after complete desiccation, Johnson *et al.* 2000). Splitting times may be more recent if there was a significant time lag between refilling of the lake and the origin and beginning of diversification of *Pundamilia*. The second best model (ΔAIC 242) was the secondary contact

model with only recent gene flow, estimated to have started 925 generations ago (~1850 years). The models of only early gene flow and of constant gene flow had very similar likelihoods (ΔAIC 2106 and 2147, with overlapping likelihood distributions, Fig. 3). The model without gene flow had a very poor fit to the data (ΔAIC 12 710, Fig. 3). These results suggest that speciation occurred in the face of gene flow. However, having only two migration matrices (early and late) is a strong simplification of the speciation process. Gene flow was likely very high when *P. nyererei* and *P. pundamilia* started to diverge and then gradually decreased as assortative mating and divergence in niche space increased, before it apparently increased again some 840 years ago. However, more individuals per species would be needed to model such additional complexity. With our current data set, different models with more than two migration matrices cannot be significantly distinguished on the basis of their likelihoods (not shown).

Next, we tested whether the patterns of genomic differentiation between the sympatric *Pundamilia* species at Makobe and at Python Islands and those between the allopatric Makobe and Python populations were most consistent with parallel, single or hybrid origin models (Figs 4 and S2, Table S4, Supporting information). We compared four demographic models differing in topology: (1) a single speciation event between *P. nyererei* and *P. pundamilia* with subsequent independent colonization of Python and Makobe Islands by both species and interspecific gene flow in sympatry at each island but no gene flow between island populations of the same nominal species, (2) a parallel speciation model with two independent events of speciation into *P. nyererei* and *P. pundamilia*, wherein one species pair occurs at Makobe Island, and the other one at Python Island, allowing for subsequent gene flow between allopatric island populations of the same nominal species but none between sympatric species, (3) a hybrid origin model, wherein the red *Pundamilia* population from Python results from introgression between the blue *Pundamilia* population from Python and *P. nyererei* from Makobe Island, allowing for recent gene flow between the species in sympatry at both islands, and (4) a model of parallel speciation, whereby the Python species are derived from a hybrid ancestor, also allowing for recent gene flow in sympatry (Fig. 4a, Table S4, Supporting information). Given that our single island analysis suggested the occurrence of early gene flow (Fig. 3), we allowed for continuous gene flow between the two-first populations after their initial split 6000 generations ago. Among these models, the single speciation model (1) had the best model fit, followed by the model of speciation at Python from a hybrid population (model 4; Fig. 4a).

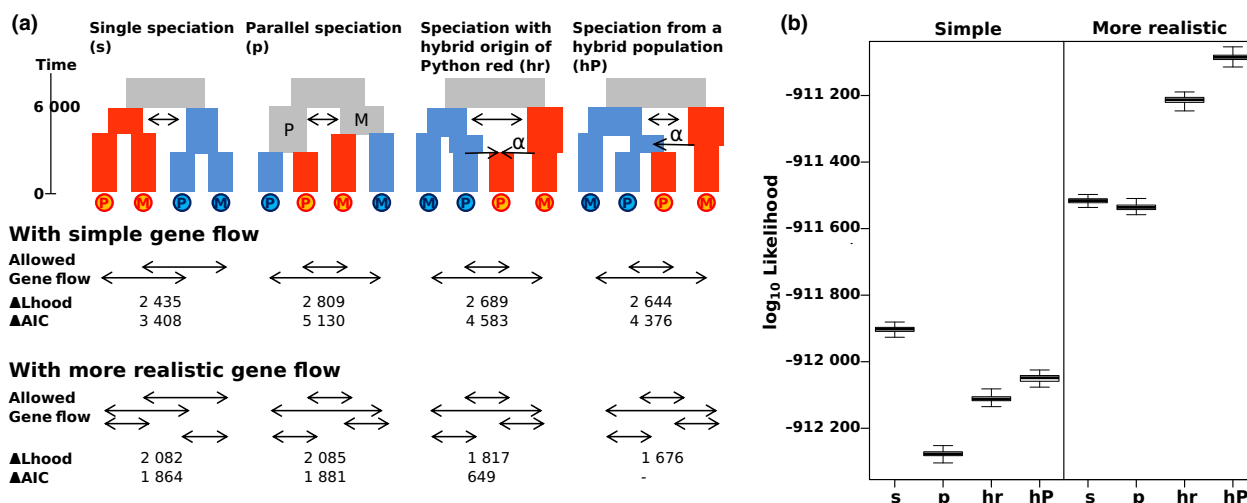


Fig. 4 Comparison of demographic models reveals highest likelihood for a model with speciation from a hybrid ancestor. (a) Illustrations of demographic models with different tree topologies (single, parallel and hybrid origin of one or both Python populations) with different gene flow scenarios (simple or more realistic). The numbers below the models denote the delta likelihoods (Δ Lhood, difference between maximum possible and obtained model likelihood in \log_{10} units) and delta AIC (Δ AIC) to the overall best model (speciation from a hybrid population at Python). (b) Boxplots showing the \log_{10} Likelihood distributions from 100 expected SFS each approximated using 1 million coalescent simulations under the parameters that maximize the likelihood for each model. For each gene flow category (simple and more realistic), the likelihoods are given for the models of single speciation (s), parallel speciation (p), speciation with hybrid origin of the Python red population (hr) and speciation from a hybrid population (hP). Other models tested and associated likelihoods are given in Fig. S2 (Supporting information).

We also tested models without gene flow between the populations resulting from the initial split (early gene flow), allowing only for recent gene flow during the last 500 generations (Fig. S2, Supporting information). Consistent with the results from the models for Makobe Island that speciation occurred in the face of gene flow (Fig. 3), demographic models with only recent gene flow during the past 500 generations had low likelihoods and are thus not discussed in detail (Fig. S2, Supporting information). Additionally, we tested models of parallel speciation whereby the two species at Python Island form the sister clade either of *P. nyererei* or of *P. pundamilia* from Makobe Island (Fig. S2, Supporting information). Again, because of very low likelihoods of these models, we do not discuss these results.

Next, we tested whether we could improve the fit with more realistic patterns of recent gene flow. We allowed for continuous migration between island populations of the same nominal species, and for continuous gene flow between the two species in sympatry (more realistic gene flow, Figs 4 and 5, Table S4, Supporting information) in addition to the early gene flow between the two populations arising from the first split 6000 generations ago. Allowing for more realistic gene flow clearly improved the model fit, particularly for the hybrid origin models. The model of speciation from a hybrid population at Python Island is the best model among all the tested scenarios (Figs 5 and S2,

Supporting information). Under this model, the first speciation event into *P. pundamilia* and *P. nyererei* sampled at Makobe is set to 6000 generations ago. The inferred parameters suggest that about 4800 generations later, *P. pundamilia* colonized the Mwanza Gulf (e.g. Python Island) and another 1000 generations later, *P. nyererei* also colonized the Mwanza Gulf and introgressed into the local *P. pundamilia* population (95% CI: 123–649 generations ago). Shortly after the introgression event (95% CI: 67–321 generations ago), the admixed population splits into two species (Fig. 5). This model suggests that the species sampled at Python Island evolved at least partially independently from those sampled at Makobe Island. We thus call the species at Python Island *P. sp.* 'nyererei-like' (red) and *P. sp.* 'pundamilia-like' (blue). The introgression event is estimated to have contributed a high proportion of the genetic variation (47%) of the ancestor of both Python species. Recent gene flow was estimated to be especially high from the red species into the blue species at both islands and from *P. sp.* 'nyererei-like' at Python to *P. nyererei* at Makobe Island (Fig. 5).

To better characterize the hybridization event, we tested alternative models of hybridization scenarios. Allowing for a second *P. nyererei* introgression event into the red Python species did not increase the likelihood and the second introgression was estimated to be very low (0.4%, Fig. S2, Supporting information). Furthermore, the model fit was lower if hybridization was

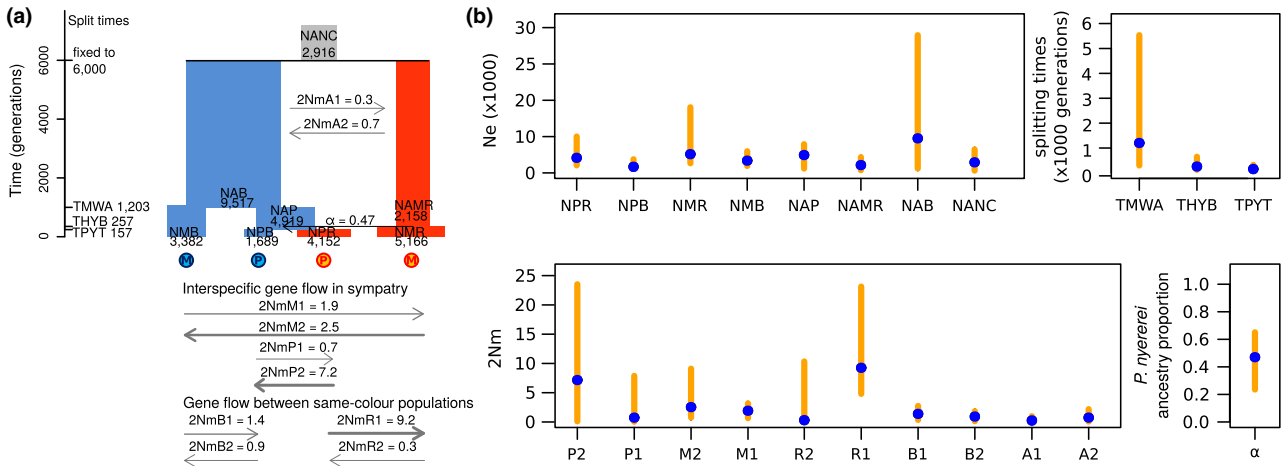


Fig. 5 The demographic model with the best fit to the observed data is a model with speciation from a hybrid ancestor giving rise to the species pair at Python Island. (a) Model illustration with parameter point estimates. Rectangles represent populations, whereas the width indicates effective population size (N) and the height corresponds to the inferred splitting times (T). THYB refers to the time of the *P. nyererei* introgression event which is shortly followed (within about 100 generations) by the split into two species at Python island at TPYT. Ancestral population sizes are labelled as ‘NA’, whereas ‘NANC’ refers to the size of the population ancestral to all sampled populations. Islands are abbreviated as ‘P’ and ‘M’ for Python and Makobe, respectively, and the nominal species as ‘B’ for *P. pundamilia* (blue) and ‘R’ for *P. nyererei* (red). The *P. nyererei* introgression event into the Python ancestor is indicated with a black arrow with ancestry proportion α . Grey arrows indicate gene flow given as effective number of haploid immigrants per generation ($2Nm$) forward in time. (b) Confidence intervals for parameter estimates based on 200 bootstrap replicates. The point estimates are shown with blue dots and the 95% confidence intervals with orange vertical bars. For parameter names, see illustration in (a).

modelled as having occurred in two separate events, one at the origin of each Python species, or if *P. nyererei* was the first species to colonize the Mwanza Gulf and speciation occurred from a hybrid population after *P. pundamilia* arrived in a second event and introgressed (Fig. S2, Supporting information).

Our parameter estimates could be biased if the four whole-genome-sequenced individuals per population were not representative of their populations. As an example, if a *P. sp.* ‘nyererei-like’ individual from Python Island was a recent hybrid, it would inflate the ancestry proportions and gene flow estimates. Therefore, we assessed the cluster proportions of the whole-genome-sequenced individuals compared with the RAD-sequenced individuals (15–21 individuals per population) in a STRUCTURE analysis and found that the ancestry proportions of the individuals used for whole-genome sequencing are representative of their respective populations (Figs S3 and S4, Supporting information). The STRUCTURE analysis further suggests that the red species at Python Island (*P. sp.* ‘nyererei-like’) has a high similarity to *P. nyererei* Makobe and that the red and blue species at both islands in the northwestern Mwanza Gulf (Python and Kissenda) are highly similar.

Discussion

Discriminating between alternative speciation and migration scenarios to explain the current distribution

of sister species in the genus *Pundamilia* is important for understanding the evolution of the Lake Victoria cichlid species flock. Here, we confirmed the findings by Seehausen *et al.* (2008) that the red and blue *Pundamilia* cichlids are genomically distinct at all islands except for the highly turbid water site, Luanso Island, and that genetic differentiation between sympatric species decreases with water turbidity (Fig. 2). Second, we found that the speciation event into red and blue *Pundamilia* occurred in the face of gene flow (Fig. 3). Third, the *Pundamilia* individuals cluster by island and by species within island rather than by nominal species across islands (Figs 2 and S1, Supporting information). Demographic modelling shows that this is best explained by two separate origins of the species pairs at Makobe and Python Islands, whereby both species at Python Island are derived from a hybrid population (Figs 4 and 5). In the following, we discuss each of these findings and the general concepts and implications. Finally, we propose a scenario for the evolution of the species complex by combining our modelling results with previous findings about mate choice and ecology of these species (Fig. 6).

Genetic clustering of individuals by species and geography

With the exception of Luanso Island, nyererei-like males with red nuptial coloration and pundamilia-like

males with blue nuptial coloration belong to distinct, yet fully sympatric genetic clusters at each island, confirming earlier results (e.g. by Seehausen *et al.* 2008; Seehausen 2009; Selz *et al.* 2016) that they represent distinct species in sympatry at each island that largely mate assortatively. Some of the individuals from Python and Kissenda Islands that are phenotypically intermediate are also genetically intermediate, but others are genetically indistinguishable from *P. nyererei* or from *P. pundamilia* individuals. This indicates that phenotypically intermediate individuals can represent backcross individuals and are not all F1 or F2 hybrids.

Consistent with previous findings (Seehausen *et al.* 2008), the genetic structure of the *Pundamilia* populations does not simply reflect the nominal species but reveals a more complex evolutionary history with gene flow. Among island populations of *P. pundamilia*, we find overall increasing genetic distance with geographical distance between islands, consistent with isolation by distance. This is not the case in *P. nyererei*, consistent with earlier work using microsatellites (Seehausen *et al.* 2008), and in line with evidence for more gene flow between islands in this species. The populations of each species at Kissenda and Python Islands in the north-western Mwanza Gulf are each genetically very similar and cluster together on PC2 and PC3, suggesting that they share a similar evolutionary history. The same is supported also by the fact that they resemble each other phenotypically, that is both *P. sp.* 'nyererei-like' populations share unique features of morphology and of female and male coloration (Table S1, Supporting information, Seehausen 1996), and both *P. sp.* 'pundamilia-like' populations have continuous broad vertical bars, whereas *P. pundamilia* from Makobe Island has broken bars interrupted by a broken midlateral and dorsolateral stripe (Table S1, Supporting information, Seehausen 1996).

The larger genetic distance between populations from Python and Luanso Islands, despite smaller geographical distance in the central and southern Mwanza Gulf, respectively, may be due to genetic drift and interspecific gene flow at Luanso Island. Luanso individuals show no signs of genetic structure between male nuptial colour morphs, nor do they show associations between nuptial coloration and multilocus (RADseq) genotype, suggesting that the Luanso individuals represent a single population with considerable colour variation. It is possible that both *Pundamilia* species independently colonized Luanso Island and admixed completely in the turbid water. This would be consistent with previous findings that female mating preferences segregate in the Luanso population, with some females having significant preferences for either red or

blue males, whereas most females do not show colour-based preferences (van der Sluijs *et al.* 2008).

Speciation with gene flow

The comparison of alternative speciation models suggests that divergence between *P. nyererei* and *P. pundamilia* occurred in the face of gene flow. Due to the simplification of gene flow to one or two different migration matrices per model, we cannot completely rule out short periods of allopatry, but we consider this unlikely given the very wide geographical and ecological distribution of *P. pundamilia* and the patchy and fully sympatric distribution of *P. nyererei* nested within that of *P. pundamilia*.

We also find evidence for recent gene flow between species in sympatry as well as between islands within nominal species with demographic modelling (Figs 4 and 5) and PCA (Fig. 2). Ongoing gene flow between sympatric species is not unexpected in a young adaptive radiation such as that of the Lake Victoria cichlids (Seehausen 2004; Grant & Grant 2008; Abbott *et al.* 2013; Lamichhaney *et al.* 2015). However, that the species remain distinct in full sympatry despite considerable interspecific gene flow implies a role for divergent or disruptive selection in sympatry (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Gavrillets 2004).

Hybrid origin of the Python Island species pair

Our demographic modelling approach suggests that speciation from a hybrid population in the Mwanza Gulf is the most likely model among all tested. If we constrain gene flow to occur among nonsister taxa populations (simple gene flow scenario), we find that the single speciation model reaches the highest relative likelihood (Fig. 4). However, our results suggest that there is considerable gene flow between allopatric populations of the same nominal species (see also Fig. 5). Indeed, if we include such migration events, the likelihood of the models increases considerably, with the speciation from a hybrid population model attaining the highest likelihood. Note that in the simple gene flow scenarios, we did not allow for migration between island populations, and that likely explains why the hybrid speciation model was not favoured in that case. Assuming that the speciation from a hybrid population model with migration among island populations is correct, by ignoring such migration rates the models are forced to reproduce the high genetic similarity between the *P. sp.* 'nyererei-like' island populations as recent shared ancestry. This finding highlights the importance of testing models with different complexities informed by observational data.

Asymmetric gene flow and effective population sizes

The higher amount of gene flow inferred to have occurred from *P. nyererei* into *P. pundamilia* than vice versa is consistent with the larger local census population sizes of *P. nyererei* (Seehausen 1996, 1997; Bouton *et al.* 1997; Seehausen & Bouton 1997). As in our models the amount of gene flow was not allowed to change over time, whereas in reality gene flow levels almost certainly were high early in speciation and then decreased as reproductive isolation strengthened, current gene flow may be lower than estimated in our models (or splitting times may be underestimated). The high gene flow estimates are supported by the presence of intermediate phenotypes at Python Island and also at Kissenda Island and are also predicted by the relatively turbid waters (Seehausen *et al.* 2008). Yet, colour-based assortative mating under clear water broad-spectrum light conditions in the laboratory is strong among individuals collected from Python Island (Haesler & Seehausen 2005; Seehausen *et al.* 2008; Selz *et al.* 2014b) and is likely important in retaining species differentiation despite some hybridization.

Allopatric gene flow between island populations likely did not occur directly from Makobe to Python or vice versa. Several islands between Makobe and Python Islands host *Pundamilia* populations and they are separated by unsuitable habitat (these species are rock specialists, and intervening habitat lacks rocks) (Fig. 1). Gene flow between the Makobe and Python populations thus likely occurred in an 'island hopping' fashion, as is known from many studies of spatial genetic structure in rock specialist cichlids (Arnegard *et al.* 1999; Wagner & McCune 2009; Koblmüller *et al.* 2011).

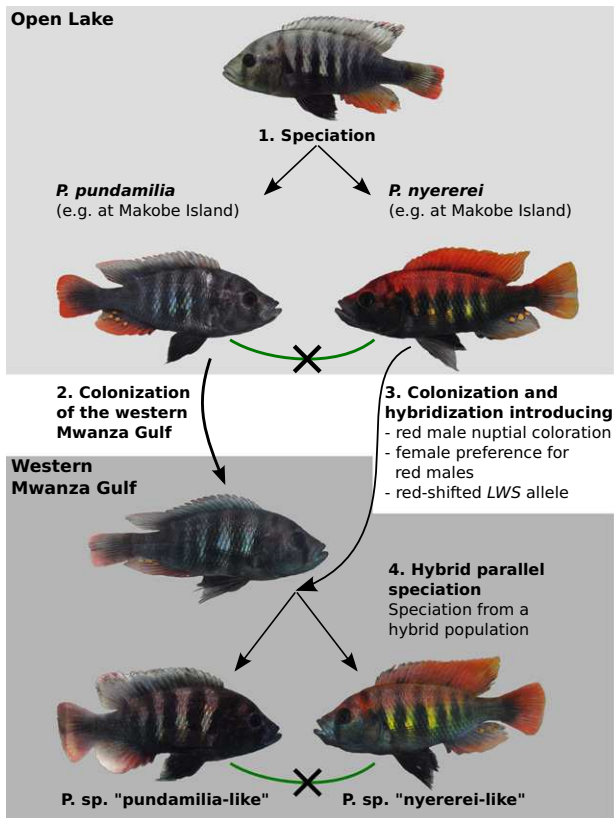
The large ancestral effective population size inferred for *P. pundamilia* is consistent with the widespread occurrence of *P. pundamilia* in Lake Victoria and indicates that the ancestral population was structured. The much smaller ancestral population size of *P. nyererei* is in line with the patchy and much smaller distribution of that species (Fig. 1, Seehausen 1996, 1997; Seehausen *et al.* 1998; Seehausen 2009). However, at each island inhabited by both species, the local census population size of *P. nyererei* is generally larger than that of *P. pundamilia*, including at Python and Kissenda Islands, where *P. sp.* 'nyererei-like' occupies the entire depth range, whereas *P. sp.* 'pundamilia-like' is more restricted to shallow water (Seehausen 1996, 1997; Bouton *et al.* 1997; Seehausen & Bouton 1997). In the best fitting model, this difference in local abundance is reflected in the larger population sizes of *P. nyererei* and of *P. sp.* 'nyererei-like' as compared to the sympatric *P. pundamilia* and *P. sp.* 'pundamilia-like' populations, respectively (Fig. 5).

'Hybrid parallel speciation' in Lake Victoria cichlids

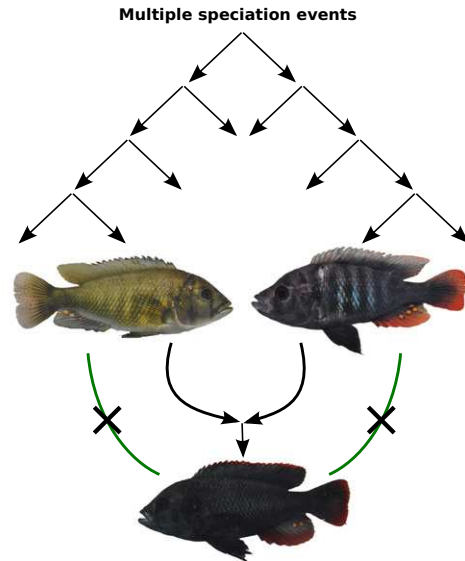
A scenario consistent with our modelling results (Figs 5 and 6) and previous findings from research on these species is one where the original speciation event that produced *P. pundamilia* (blue) and *P. nyererei* (red) happened outside the Mwanza Gulf several thousand generations before the Mwanza Gulf was colonized by *P. pundamilia* but after the refilling of the lake 14 600 years ago. Eventually, about 1000 generations after *P. pundamilia* had established populations in the Mwanza Gulf, massive introgression occurred from *P. nyererei* into the *P. pundamilia* population resulting in a hybrid population with similar ancestry proportions of *P. nyererei* and *P. pundamilia*. This may have happened through immigration of *P. nyererei* individuals from islands outside the Mwanza Gulf, which then hybridized with the local *P. pundamilia*.

Red coloration is dominant in laboratory crosses between the species (Magalhaes *et al.* 2009). As the red coloration of initial *P. nyererei* immigrants and their F1 hybrid offspring would have made for more conspicuous visual signals than blue in deeper water (Seehausen *et al.* 1997), and their red-shifted colour vision would have affected both mate preference and facilitated vision in deeper water (Maan *et al.* 2006), *P. nyererei* alleles underlying these traits would have immediately had adaptive value in the Mwanza Gulf and would probably have allowed the introgressed population of *Pundamilia* to expand into greater water depth and persisted in the deeper sections of the habitat. Consistent with this hypothesis, contemporary populations of *P. sp.* 'nyererei-like' at Python and Kissenda Islands have nearly fixed the red-shifted LWS opsin allele (Seehausen *et al.* 2008) and are most abundant at depths greater than 2 m, whereas *P. sp.* 'pundamilia-like' has the blue-shifted LWS allele and is most abundant at depths less than 2 m (Seehausen 2009). Assortative mating seems to be mainly due to divergent female preferences for red and blue male nuptial coloration (Stelkens *et al.* 2008; Selz *et al.* 2014b), which have a relatively simple genetic basis (Haesler & Seehausen 2005). As male Lake Victoria cichlids often bias aggression towards males of their own colour or the locally more common colour phenotypes (Seehausen & Schluter 2004) and additionally *P. nyererei* males dominate *P. pundamilia* males in dyadic interactions (Dijkstra *et al.* 2005), the first *P. nyererei* males migrating to islands in the Mwanza Gulf, and their red F1 hybrid offspring, may have had an advantage over the local *P. pundamilia* males in competition for territories, which would facilitate reproductive success. The interaction of sexual and natural selection acting on the same set of traits may

Hybrid parallel speciation



Classical hybrid speciation



A geographically restricted pulse of hybridization (e.g. due to local habitat disturbance or secondary contact) leads to the formation of a hybrid population. Selection purges incompatible combinations of reproductive isolation loci forming a hybrid species with incompatibilities to both parental lineages. New combinations of ecologically relevant traits and/or transgressive traits allow the occupation of a new niche, and new combinations of mating cues and preference alleles lead to assortative mating in the emerging hybrid species.

Fig. 6 Illustration of the proposed evolutionary scenario of hybrid parallel speciation and a comparison to classical hybrid speciation. A scenario consistent with our results is one of initial speciation from a blue ancestor into *P. pundamilia* and *P. nyererei* (here represented by the populations at Makobe Island, M), followed much later by the colonization of the Mwanza Gulf (including Python Island, P) by *P. pundamilia*. Later again *P. nyererei* arrive in the Mwanza Gulf, hybridize with the local *P. pundamilia* population and merge into a hybrid population that carries a mixture of genetic variation from both species. This hybrid population speciates again into sympatric *P. sp.* 'pundamilia-like' and *P. sp.* 'nyererei-like' at Python (illustrated by the crossed green lines, Selz, *et al.* 2014b) and also some evidence for assortative mating between individuals of *P. sp.* 'nyererei-like' from Python and individuals of the original *P. nyererei* from Makobe Island (Selz *et al.* 2016). 'Hybrid parallel speciation' relies on the local availability of the ecological niches of both the local and the geographically distant parental species giving hybrids with alternative trait combinations fitness advantages in different parts of the ecological range of the hybrid population. Should the geographical ranges of *P. sp.* 'nyererei-like' and *P. nyererei* ever expand sufficiently to meet, the ecological and phenotypic similarity of these species may predict that the species merge into a single species. In contrast, classical hybrid speciation (right panel) produces one species of hybrid origin that may be ecologically distinct and reproductively isolated from both parental species and can hence coexist in sympatry with both. We exemplify hybrid speciation in Lake Victoria cichlids with *Mbipia mbipi* which has resulted from hybridization between *M. lutea* and *P. pundamilia* (Keller *et al.* 2013). Larger evolutionary distance between the parental species (illustrated by multiple intermittent speciation events) may facilitate the formation of a hybrid species as more incompatibilities are expected to segregate in the hybrid population, and the novel ecological and/or sexual trait combinations may be more transgressive and differ from both parental species along more phenotypic axes of divergence (Selz *et al.* 2014a,c). Hybrid speciation requires either an underutilized niche that the hybrid species can exploit better than either parental species or geographical distance to the parental species. In the case of 'hybrid parallel speciation', on the other hand, two species are generated from a hybrid population that are reproductively isolated and ecologically distinct from each other but that are expected to be ecologically and phenotypically similar to the two parental species. Hybrid speciation and 'hybrid parallel speciation' represent different outcomes of a continuum of possible ways by which admixture variation may be involved in speciation, varying in extent of ecological differentiation and reproductive isolation within the hybrid population and between the hybrid population and its parental species, mediated by the geographical context, niche availability and the genetic similarity between the parental species.

subsequently have driven the evolution of a new species pair from within the admixture-enriched local *Pundamilia* population.

Even though we only tested the populations at Python and Makobe Islands, given that populations of the same colour at Python and Kissenda Islands mostly cluster

together in the PCA (Fig. 2b), exhibit low F_{ST} (Fig. S1, Supporting information) and share the same phenotypic traits (Table S1, Supporting information, Seehausen 1996), likely all *Pundamilia* populations at both islands and potentially at all islands in the northwestern Mwanza Gulf (Fig. 1b) are derived from the same hybrid speciation event. We note that our current data and analyses do not allow us to clearly distinguish between a scenario whereby red and blue species re-emerged in the Mwanza Gulf after complete mixing of the ancestral *P. pundamilia* population with the late colonizing *P. nyererei*, or if some LD among *P. nyererei* alleles and among *P. pundamilia* alleles was retained and facilitated rapid re-emergence of nyererei-like and pundamilia-like populations. Functional analyses and genome scans may help to discriminate between these alternatives.

To be considered a case of classical hybrid speciation, a hybrid population needs to evolve reproductive isolation from both parental lineages soon after the admixture event (Arnold 1997; Mallet 2007; Abbott *et al.* 2010), and that does not appear to be the case in the situation studied here. On the other hand, parallel speciation as defined by Schluter & Nagel (1995) predicts that ecologically similar species which evolved in parallel lack reproductive isolation but show reproductive isolation to ecologically different species in sympatry. The *Pundamilia* species we studied here rather fulfil the expectations from parallel speciation with weaker reproductive isolation among allopatric species of similar phenotype and ecology than between sympatric species. *P. sp.* 'nyererei-like' females at Python Island strongly prefer own-type males over sympatric *P. sp.* 'pundamilia-like' males (Selz *et al.* 2014b) which closely resemble the *P. pundamilia* parental lineage. The traits crucial for assortative mating (red male coloration, preference for red, and red-shifted *LWS* alleles; Seehausen *et al.* 2008; Selz *et al.* 2014b) are likely derived from the second parental lineage, *P. nyererei*, from outside the area (Fig. 6). Reproductive isolation between *P. sp.* 'nyererei-like' at Python Island and the *P. nyererei* from Makobe Island is less straightforward, as both exhibit the same general type of male nuptial coloration, namely a bright red dorsum and dorsal fin and yellow flanks. However, as explained above, they are distinct in some other elements of nuptial coloration, such that the chroma of colour of different body and fin regions significantly differs between *P. nyererei* and *P. sp.* 'nyererei-like' (Table S1, Supporting information, Seehausen 1996; Castillo Cajas *et al.* 2012). There is also recent experimental evidence for behavioural reproductive isolation between *P. sp.* 'nyererei-like' from Python and *P. nyererei* from Makobe (Selz *et al.* 2016). In the case of *P. sp.* 'pundamilia-like', females prefer

conspecific males over heterospecific *P. sp.* 'nyererei-like' males (Selz *et al.* 2014b), but whether they also prefer *P. sp.* 'pundamilia-like' males over *P. pundamilia* males awaits further testing.

However, *Pundamilia* are not a classical case of narrow-sense parallel speciation, because the evolutionary histories between the two red-blue species pairs are not completely independent (first criterion of Schluter & Nagel 1995). This system may represent a special case of broad-sense parallel speciation initiated by introgressive hybridization between a pair of precursor species, which we term 'hybrid parallel speciation' (Fig. 6). It is conceivable that the species from Python Island would merge with their allopatric parental populations of similar colour (e.g. from Makobe Island) if they ever meet upon range expansion, much like the prediction for ecological parallel speciation (Schluter & Nagel 1995). Therefore, 'hybrid parallel speciation' may not necessarily increase the total number of lasting biological species, but may often rather generate species with polyphyletic origins. If such parallel speciation events were common, the already exceptionally high speciation rate in Lake Victoria cichlids may have been yet underestimated.

In the 'transporter hypothesis' (Schluter & Conte 2009), parallel evolution of a particular type of species is also facilitated by gene flow from a precursor species of similar type. This hypothesis was introduced to describe the parallel evolution of freshwater stickleback from marine stickleback in different freshwater catchments. Here, after the first origin of a freshwater-adapted stickleback population, freshwater-adapted alleles were introduced into the marine population through gene flow followed by multiple generations of recombination into marine genomic backgrounds (Schluter & Conte 2009). Upon colonization of a new river system from the Ocean, many freshwater alleles were recruited again through selection and freshwater genotypes are reassembled (Schluter & Conte 2009). We think that hybridization had a more direct effect in *Pundamilia*, as our high estimate of *P. nyererei* ancestry in the ancestral population of Python Island before speciation (47%, Fig. 5) suggests that a large fraction of the genomic variation introgressed from the parental *P. nyererei* lineage. This increases the likelihood that linkage among parental alleles was retained to some extent in the hybrid population, which would facilitate rapid speciation in the absence of geographical isolation and might affect the rate at which the hybrid species stabilize and also the extent of parallelism with the original species pair.

Hybrid speciation has been hypothesized to underlie the origins of *Mbipia mbipi* and *Pundamilia sp.* 'pink anal fin' from Lake Victoria (Keller *et al.* 2013), and given that

the same or very similar male nuptial colour phenotypes (e.g. blue, red-back, red-belly) define sympatric sister species in most genera of Lake Victoria cichlids (Seehausen *et al.* 1999), shuffling of the underlying alleles between nonsister species by occasional or geographically localized hybridization, followed by speciation, may have happened repeatedly. As the endemic species in Lake Victoria evolved in only 15 000 years (Stager & Johnson 2008), it is unlikely that the alleles underlying these colour patterns arose repeatedly from de novo mutations, but rather form part of the standing genetic variation shared by many Lake Victoria cichlids. However, strong sexual selection is expected to deplete the standing variation at genes relevant to female preference and male nuptial coloration within each species (Seehausen 2004; Barrett & Schluter 2008). Standing genetic variation in key speciation traits may be recovered through occasional interspecific gene flow in adaptive radiations (syngameon hypothesis, Grant & Grant 1992; Seehausen 2004), and interspecific gene flow has been demonstrated in a large number of adaptive radiations including *Heliconius* butterflies (Gilbert 2003; Mavárez *et al.* 2006; Jiggins *et al.* 2008; Mallet 2009; The Heliconius Genome Consortium 2012), Darwin's finches (Grant & Grant 1994; Grant *et al.* 2005; Lamichhaney *et al.* 2015), *Mimulus* monkeyflowers (Stankowski & Streisfeld 2015), *Rhagoletis* fruit flies (Feder *et al.* 2005), sunflowers (Rieseberg *et al.* 2003), clownfishes (Litsios & Salamin 2014), sailfin silversides (Herder *et al.* 2006) and cichlids of Lakes Tanganyika (Weiss *et al.* 2015), Malawi (Genner & Turner 2012), Victoria (Keller *et al.* 2013) and Barombi Mbo (Schliewen & Klee 2004). More work is needed to make a more direct link between interspecific gene flow and diversification in Lake Victoria cichlids. Importantly, identifying the genomic regions under divergent selection or involved in reproductive isolation between species would allow assessment of the relative importance of introgression as a source of genomic variation of relevance to speciation.

Conclusions

Here, we show how demographic modelling can improve our understanding of the processes underlying the observed patterns of genetic similarity and dissimilarity in a complex of closely related cichlid species from Lake Victoria. We provide evidence that similar *Pundamilia* sister species pairs at different islands have arisen twice, where the second event is associated with introgressive hybridization between a pair of precursor species. Our study is consistent with a role of interspecific gene flow in the diversification of Lake Victoria cichlids, as proposed in the syngameon hypothesis for adaptive radiations. Further

investigations should target the key mechanisms in the diversification of *Pundamilia*, the genes and genomic architecture underlying the relevant traits and their evolutionary histories.

Acknowledgements

We thank the Tanzanian Commission for Science and Technology for research permission, and the Tanzanian Fisheries Research Institute for facilities and logistical support. We are indebted to Mhoja Kayeba and Mohammed Haluna for technical assistance in the field and to Inke van der Sluijs and Nellie Konijnendijk for collecting some of the tissue samples used in this study. We also thank Marcel Häsler and Salome Mwaiko of the Department of Fish Ecology and Evolution at EAWAG, Keith Harshman of the Lausanne Genomic Technologies Facility, and Cord Drögemüller, Tosso Leeb, Muriel Fragnière and Michèle Ackermann of the NGS platform of the University of Bern for Illumina sequencing and laboratory support. We thank Stefan Zoller from the Genetic Diversity Center (GDC) at ETH Zürich for bioinformatics support. Genomic analyses were performed using the computing infrastructure of the GDC, the Euler computer cluster at ETH Zurich and the Ubelix computer cluster at University of Bern, Switzerland. This research was supported by the Swiss National Science Foundation grant PDFMP3 134657 to OS and LE.

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Data accessibility

Demultiplexed fastq files of RADseq and whole-genome data sets have been deposited at NCBI SRA under BioProject PRJNA339828 (<http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA339828>) and BioSamples SAMN05711156–SAMN05711318. Input files used for fastsimcoal2 and observed site frequency spectra are available on Dryad under doi:10.5061/dryad.2jd45.

O.S. and J.I.M. designed the study; O.M.S. collected, and O.S. and O.M.S. identified the cichlid samples; J.I.M. performed the laboratory work and conducted the analyses, with assistance from O.S., V.C.S., L.E., D.A.M. and C.E.W. J.I.M. wrote the manuscript with contributions from O.S., L.E., C.E.W., D.A.M., V.C.S. and O.M.S.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Pairwise divergence (F_{ST}) between all eight populations of red and blue *Pundamilia* at Makobe (M), Kissenda (K), Python (P), and Luanso (L) Islands.

Fig. S2 Demographic models allowing only for recent gene flow in the past 500 generations.

Fig. S3 STRUCTURE analysis suggests that the individuals used for whole-genome sequencing are representative of their respective populations.

Fig. S4 Mean likelihood over 10 replicate STRUCTURE runs for each K value.

Table S1 Summary of phenotypic and ecological differences between *Pundamilia pundamilia* and *P. nyererei*.

Table S2 Information on individuals used for RAD sequencing and whole-genome sequencing including missing data proportions and mean sequencing depth.

Table S3 Search ranges and highest likelihood values for the parameters of the speciation with gene flow models.

Table S4 Search ranges and highest likelihood parameter values of the demographic history models with more realistic gene flow.