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# The genetics of mate preferences in hybrids between two young and sympatric Lake Victoria cichlid species

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The genetic architecture of mate preferences is likely to affect significant evolutionary processes, including speciation and hybridization. Here, we investigate laboratory hybrids between a pair of sympatric Lake Victoria cichlid fish species that appear to have recently evolved from a hybrid population between similar predecessor species. The species demonstrate strong assortative mating in the laboratory, associated with divergent male breeding coloration (red dorsum versus blue). We show in a common garden experiment, using DNA-based paternity testing, that the strong female mate preferences among males of the two species are fully recovered in a large fraction of their F2 hybrid generation. Individual hybrid females often demonstrated consistent preferences in multiple mate choice trials (more than or equal to five) across a year or more. This result suggests that female mate preference is influenced by relatively few major genes or genomic regions. These preferences were not changed by experience of a successful spawning event with a male of the non-preferred species in a no-choice single-male trial. We found no evidence for imprinting in the F2 hybrids, although the F1 hybrid females may have been imprinted on their mothers. We discuss this nearly Mendelian inheritance of consistent innate mate preferences in the context of speciation theory.

## 1. Introduction

Behavioural assortative mating is considered to play a significant role in the origin and maintenance of reproductive isolation among species [1,2]. The rate of and constraints to the evolution of behavioural assortative mating is probably often influenced by the genetic architecture of mate preferences and the nature and strength of genetic and non-genetic influences, such as imprinting and experience. For example, modelling studies suggest that sympatric and parapatric speciation starting from a monomorphic population is more probable in cases where assortative mating or female preference among male courtship genotypes is influenced by relatively few genetic loci [3–5], although models starting from large standing variation may not have this constraint of preference architecture [6]. However, a small number of preference genes tends to facilitate speciation in many models of speciation with gene flow [7,8]. Empirical studies of the genetics of species divergence in mating preferences are still rare. Some of the empirical results are consistent with few genes having a major effect on female assortative mating in cichlid fish and *Heliconius* butterflies [9–12]. In other systems, mostly insects, female choice appears to have a more quantitative genetic background [13–15].

The Lake Victoria rocky-shore cichlid fishes of the genus *Pundamilia* have emerged as a significant model system for the study of speciation, being representatives of a spectacular hyperdiverse, rapid adaptive radiation and being relatively tractable as a laboratory species for breeding and mate choice experiments [16,17]. Following the completion of their genome sequence [18], the evolutionary history of focal populations in the southeast part of the lake has been reconstructed [19]. Analysis of genome-wide sequence data indicates that the species with red dorsum (*P.* 'nyererei-like') and blue (*P.* 'pundamilia-like') males at Python Island have recently diverged *in situ*, following a period of massive introgression with resident *P. pundamilia* on the colonization of the island by *P. nyererei* from elsewhere in the lake [19].

The *Pundamilia* species, like other haplochromine cichlid fishes, show strong sex role differentiation and associated sexual dimorphism: the smaller, cryptic females are mouthbrooders, caring for the offspring for several weeks, while the larger brightly coloured males defend territories and display to attract females, but play no part in rearing the offspring [20]. Such a breeding system is likely to generate strong sexual selection acting through male–male competition and female preference for male courtship traits [21]. Closely related haplochromine species often differ markedly in male nuptial colour and it has been proposed that this is associated with divergent female mate preferences [22], which have been demonstrated in a number of experimental trials [23–25]. The resultant assortative mating between females with a certain preference and males expressing the corresponding trait may play a significant role in the maintenance and perhaps sometimes the origin of reproductive isolation among sympatric species [16].

In the *Pundamilia* red/blue system, increasing water depth is associated with differentiation in alleles at the long wavelength sensitive opsin gene (*LWS*), female preferences and male nuptial colour, and it is likely that the sensory environment along this microhabitat gradient has influenced divergence through a process of 'sensory drive' [26]. Of course, mating signals are often multimodal and subject to multivariate selection [27–29] which is most likely also the case in *Pundamilia* [16,17,30]. However, in the *Pundamilia* system, female preferences for male nuptial coloration—itself likely to be oligogenic [31]—appear to be necessary and sufficient for assortative mating [30,32,33].

In haplochromine cichlids, trait segregation in F2 hybrids has been shown for female preferences [9,12], male nuptial coloration [12,31,34] and male attractiveness to parental species [33,35]. This includes the *Pundamilia* system, where, furthermore, studies suggest an absence of physical linkage between male nuptial colour and female mate preference [36]. At Python Island, gene flow between the species is estimated to be ongoing [19]. Therefore, the observed strong linkage disequilibrium between male colour and female preference is likely to be maintained by divergent selection. A behavioural study on the second-generation (F2) hybrid offspring of *P.* sp. 'pundamilia-like' and *P.* sp. 'nyererei-like' crosses by Haesler & Seehausen [9] revealed that female mate preference has a genetic basis, and that there may be as few as one to five major genes that contribute to the variation in this trait. That study, however, used a behavioural assay to measure mate choice, which may not be entirely predictive of actual mating decisions. Here, we used a 'common garden' approach with full-contact spawnings to examine female mate choice decisions in first- and second-generation hybrids (F1 and F2). Wild-type females were included as a control. We used molecular paternity determination to measure directly the mating decisions of females in the laboratory [24] and examined the repeatability (more than or equal to five spawning decisions) of female mate choice over a year or more to estimate the segregation of mate preferences in the F2 hybrids of the sympatric sister species of *Pundamilia* from Python island. In contrast with Haesler & Seehausen [9], we examined if mate preferences are consistently maintained across many spawning events (the full cycle from spawning to egg maturation).

If female preference is a polygenic quantitative trait with an additive genetic basis, F2 hybrid preferences are expected to be distributed in a Gaussian-like fashion with few individuals expressing significant preferences in the tails of the distribution. By contrast, for a polygenic trait with strong dominance effects, the preference distribution of the F2 will be skewed towards either end of the distribution [37–39]. On the other hand, if preferences are not genetically determined, the preference distribution of F2 females is predicted to be more uniform across F2 females given that individuals shared the same common environment. However, in the case of gene flow, linkage disequilibrium between alleles in a polygenic trait will be broken up by recombination [40,41] and polygenic mating preferences will be difficult to maintain under such conditions. Because ongoing gene flow and recombination [17,19,26] have been shown in this young [19] species pair, and because differentiation in polygenic mating preferences will be difficult to maintain under such conditions, we predicted mate preferences to segregate as an oligogenic trait in a nearly Mendelian fashion.

## 2. Material and methods

### (a) The experimental fish

We used the sympatric sister species *Pundamilia* sp. 'pundamilia-like' and *Pundamilia* sp. 'nyererei-like' (*sensu* Meier *et al.* [19]). These taxa show a striking difference in male nuptial colours: *P.* sp. 'pundamilia-like' males are grey or pale yellow on the flanks between black vertical bars and have a metallic blue spinous dorsal fin, whereas *P.* sp. 'nyererei-like' are orange on the dorsum, dorsal head surface and dorsal fin and yellow on the flanks between black vertical bars. It is estimated that there is currently a low to moderate level of gene flow between the taxa at Python Island (the effective number of haploid immigrants per generation (2 Nm, method: forward in time) is 0.7 from *P.* sp. 'pundamilia-like' to *P.* sp. 'nyererei-like' and 7.2 in the opposite direction [19]). Species differences in female mate choice and divergent alleles at the *LWS* opsin gene are not completely fixed [17] and males with intermediate coloration are found [26]. By contrast, at Makobe Island in the open lake the sympatric species pair *P. pundamilia* and *P. nyererei* shows stronger genome-wide differentiation, is more ecologically differentiated, intermediate phenotypes are not observed and no mismatches have been reported between male coloration and *LWS* opsin allele [17,19,26]. Both species are diploid and have 22 chromosomes ($2n = 44$) [18].

Wild-type females and two F1 hybrid families (one in each cross direction) used in the mate choice experiment were bred from wild-caught parents. The fry were raised in stock tanks until large enough to be tagged with an integrated transponder (PIT tag), to enable individual identification. Using microsatellite DNA parentage analyses, we concluded that the 15 *P.* sp. 'pundamilia-like' females originated from three wild mothers and one wild sire and the six *P.* sp. 'nyererei-like' females from three to six wild mothers and five wild sires (electronic supplementary material, tables S3 and S4).

The two F2 families used in the mate choice experiment were bred from a laboratory stock collected in 1992 [42]. The F1 families were bred from the second to third laboratory generation. The F2 generations were bred by holding one F1 male (no replacement, $N = 3$) together with not more than 10 F1 females in the same aquarium. One F2 half-sib family (PN1–33) was bred from fish from two F1 families bred in 1999 from a female *P.* sp. 'pundamilia-like' x male *P.* sp. 'nyererei-like', and vice versa. This was the same F2 family used by Haesler & Seehausen [9]. The F2 broods were kept separate and hence some broods in the electronic supplementary material, figure S3 may have had the same mother, whereas we know which of the two males was the father. The other F2 family (PN34) was bred from fish from one F1 family bred in 2001 from a female *P.* sp. 'pundamilia-like' x male *P.* sp. 'nyererei-like'. The offspring were pooled into the same aquaria and hence the father is known but not the brood or mother. When F2 offspring were large enough, they were PIT-tagged and pooled into the same aquaria. The breeding set-up is given in the electronic supplementary material, figure S1.

All females had been brooded in the mouth of their mothers until independently feeding and were then raised apart from their mothers. In the data analyses, we have included all spawning wild-type and F1 females and the 69 F2 females with more than or equal to five spawning decisions in the experiment. Spawning decisions of females with less than or equal to spawning decisions are given in the electronic supplementary material, figure S3 and table S1) and were also used in the calculations of paternal and brood effects.

## (b) Mate choice

Mate choice was tested using a 'partial partition' design [24]. An aquarium measuring L 600 cm × W 80 cm × H 40 cm was divided into 10 equally sized compartments by plastic grids, eight containing one male each, four of each species. Identical halved flower pots ($D = 270$ mm, $L = 220$ mm) served as the focal point in male territories. Two chambers were accessible to females only. We used several males of each species to decrease the chance that effects of individual variation in male attractiveness could override female mating preferences for males of one species or the other. The mesh size of the plastic grids was adjusted to confine males in their compartments, but to allow the smaller females to pass through. The complement of males was replaced every second month and the female-only compartments were relocated. In total, 11 wild-caught and eight laboratory-bred *P.* sp. 'pundamilia-like' males and 11 wild-caught and six laboratory-bred *P.* sp. 'nyererei-like' males were used in the experiment (electronic supplementary material, table S3). Wild-type females were tested as a control that species-specific mating preferences would be expressed in this set-up. All females were tested with wild-type males; hybrid males were not used in these experiments.

To test whether experience altered mating preferences, 16 F2 hybrid females that had spawned six broods each and whose preferences were hence known were isolated in a tank with a male of the non-preferred species. The five *P.* sp. 'pundamilia-like'-preferring females had spawned 90–100% with *P.* sp. 'pundamilia-like' (mean = 98%), and the 11 *P.* sp. 'nyererei-like'-preferring females had spawned 83–100% with *P.* sp. 'nyererei-like' (mean = 96%). The females that subsequently spawned with a male of the species they had not preferred ($N = 9$) were allowed to brood fry until final release and potential independence of the fry. Thereafter, they were released back into the experimental tank and allowed to spawn again with a choice of males.

All experimental fish were marked with PIT tags and a small piece of the dorsal fin was cut to provide a DNA sample. Females with eggs were placed in a separate aquarium until the eggs hatched. All larvae/juveniles were euthanized using MS-222 (tricaine methanesulfonate) and stored in 95% ethanol prior to paternity analyses. All females were released back into the experimental tank after handling.

## (c) Paternity analyses

The experiment lasted 2.5 years. Five embryos from each brood were genotyped at two to five microsatellite loci, Ppun5, Ppun7, Pun17, Ppun21 and Ppun32. Methods for DNA extraction and PCR reactions were as described previously [33] with additional optimizations for multiplex analyses. The amplified DNA samples were genotyped on a Beckman Coulter CEQ 8000 capillary sequencer. Genotypes were received from the CEQ 8000 Series Genetic Analysing System 8.0.52. Paternities were determined by direct inspection of the allele size estimates on a spreadsheet, and males that possessed two alleles in a microsatellite locus that were not present in the offspring were excluded as a possible father (electronic supplementary material, tables S1–S4). We used the number of spawning decisions in figures and statistical calculations, i.e. if a brood was confirmed to be fathered by more than one male, each male was considered to be a spawning decision. F2 females in the analysed data had four to eight broods each and 5–15 spawning decisions. The complete datasets of the wild-type females, F1 hybrid females, F2 hybrid females and the males used in the experiment are included in the electronic supplementary material, figures S2 and S3 and tables S1–S3. We also provide pictures of the F2 hybrid males from PN1-33 in electronic supplementary material, figure S4.

## (d) Statistics

When analysing between-group preferences (*P.* sp. 'pundamilia-like versus *P.* sp. 'nyererei-like'; F1 hybrid females with *P.* sp. 'pundamilia-like' mother versus F1 hybrid females with *P.* sp. 'nyererei-like' mother), we, for each female, subtracted the number of spawning decisions with males of *P.* sp. 'nyererei-like' from the number of spawning decisions with males of *P.* sp. 'pundamilia-like' and analysed the differences with Mann–Whitney *U*-tests.

Within-group preferences were analysed with Wilcoxon signed ranks tests on the individual's number of spawning decisions with *P.* sp. 'pundamilia-like' and *P.* sp. 'nyererei-like'. In one F1 hybrid family, a binomial test was used due to the low number of spawning decisions per female. The preference of individual F2 hybrid females were also analysed with binomial tests. We could not estimate individual female preferences of wild-type and F1 hybrid females given the small number of decisions obtained from each female.

To test whether the F2 hybrid female spawning patterns deviated significantly from random, we simulated a distribution of spawning decisions of the 69 females that had more than or equal to spawning decisions with either a *P.* sp. 'pundamilia-like' (*Pp*) or a *P.* sp. 'nyererei-like' (*Pn*) male. To express the level of deviation from randomness, we calculated the consistency of an individual's mate choice and calculated the repeatability ($R$) of a female's spawning decisions. In quantitative genetics, the repeatability can be used to determine the upper-bound estimate of the broad sense heritability ($H^2 = V_G/V_P$) (p. 136–138, [37]). The broad sense heritability indicates the relative proportion of total phenotypic variation of a trait ($V_P$) that has a genetic basis ($V_G$). Repeatability is an upper-bound estimate of this heritability, given that similarity in a trait value (in this case, consistent preference for males of one of the two species) can both have a genetic and an environmental basis (e.g. a given female may prefer males of a given species due to previous experiences). The model assesses the extent to which a female's first spawning decision can predict her subsequent decisions, as this informs us on how strong a mate preference has been expressed. In other words, the model assesses how significantly the pattern of spawning decisions deviates from a random pattern (i.e. no preference) when analysed across all F2 females at the population level. In the simulations, each female is given a probability of mating with a *Pp* or a *Pn* male equivalent to the proportion of *P.* sp. 'pundamilia-like' and *P.* sp. 'nyererei-like' spawning decisions observed. This probability determines her first spawning decision. However, once a female has been allocated a mate preference, the strength with which this preference continues to affect subsequent spawning decisions is given by the following formulae:

$$P(x_i = Pp)Pp + R(1 - Pp),$$
$$P(x_i = Pn) = Pn + R(1 - Pn).$$

Here, $P(x_i = Pp)$ and $P(x_i = Pn)$ are the probabilities of spawning with a *Pp* and a *Pn* male at the *i*th spawning decision ($i > 1$), and *Pp* and *Pn* are the observed proportions of spawning decisions (across the entire population) with a *P.* sp. 'pundamilia-like' and a *P.* sp. 'nyererei-like' male, respectively. *R* is the repeatability coefficient ($0 \leq R \leq 1$). With $R = 0$, spawning is 'random' and proportional to the observed proportion of *Pn* and *Pp* spawning decisions. In this case, female choice will switch randomly between *Pp* and *Pn* males. With $R = 1$, however, spawning choice is fixed and all spawning decisions are for males of the same species as the first choice. In this case, females will consistently choose either a *Pp* or a *Pn* male. With intermediate values of *R*, there is a preference for a species of male, but this preference will not completely determine a spawning decision.

Furthermore, we also calculated if the number of individuals with preference for one species differed from random. When categorizing female preference for males of either one of the two species we used binomial tests and $\alpha = 0.05$ for the dataset that included females with more than or equal to six spawning decisions.

To address potential parental and brood effects, all 100 F2 females were divided into two categories: majority of spawnings with *P.* sp. 'pundamilia-like' and majority of spawnings with *P.* sp. 'nyererei-like'. Four females were omitted because they spawned equally many times with males of the two species leaving 96 females (see electronic supplementary material, figure S3). We used binomial tests to ask if the female offspring of each of the three F1 fathers were biased in their spawning decisions towards one of the two species, and $\chi^2$ to test if there was a difference between F2 females fathered by different F1 males. When analysing the brood effect, we restricted the analyses to the six broods with more than or equal to four F2 females and performed 36 pairwise Fisher exact test comparisons and Bonferroni correction to correct for multiple comparisons.

Statistics were performed in SPSS v. 23. The individual-based model was constructed in Minitab 12.1.

## 3. Results

### (a) Wild-type females spawned with their own species

There was a significant difference in spawning decisions between females of the two species (Mann–Whitney *U*-test, $n = 20$, $U = 0.00$, $p < 0.001$; electronic supplementary material, figure S2a). The *P.* sp. 'pundamilia-like' females had one to three spawning decisions each (median 2), and 14 out of 15 spawned only with conspecific males. One female mated once with *P.* sp. 'nyererei-like' and twice with conspecific males (Wilcoxon signed ranks test $T = 0$, $n = 15$, $p < 0.001$). The *P.* sp. 'nyererei-like' females also had one to three spawning decisions each (median 3), and all six spawned only with conspecific males (Wilcoxon signed ranks test $T = 0$, $n = 6$, $p = 0.024$).

### (b) F1 hybrid females generally spawned with the species of their mother

There was a significant difference in spawning decisions between the two F1 hybrid families (Mann–Whitney *U*-test, $n = 16$, $U = 2.50$, $p = 0.002$, electronic supplementary material, figure S2b). This was caused by F1 hybrid females spawning more often with the species of their mothers (*P.* sp. 'pundamilia-like' mother, two to three spawning decisions per female, median 2; two females spawned with both species, nine with *P.* sp. 'pundamilia-like' only, $N = 11$, Wilcoxon signed ranks test, $z = 45$, $p = 0.004$, *P.* sp. 'nyererei-like' mother, one spawning decision each, all spawned with *P.* sp. 'nyererei-like', two tailed Binomial test, $n = 5$, $p = 0.063$).

### (c) F2 hybrid spawning consistency suggests innate mating preference

When including females with more than or equal to six spawning decisions and $\alpha = 0.05$, 41 out of 59 F2 hybrid females had a significant preference for males of one of the two species, whereas we would have expected less than three if females mated randomly (Fisher exact test, $p < 0.001$; figure 1). The simulation model showed that the pattern of spawning decisions significantly deviated from a random pattern when analysed at the population level. Spawning preferences segregated in an almost Mendelian fashion and the majority of the females repeatedly spawned with one of the two species (figure 1). The model estimates a repeatability of spawning decisions of $R = 0.7$ (figure 2), which indicates that in our F2 population, 70% of the variation in spawning decisions is explained by actual female mate preference.

To address potential parental effects, all 100 F2 hybrid females (electronic supplementary material, figure S3) were divided into two categories: majority of spawnings with *P.* sp. 'pundamilia-like' and majority of spawnings with *P.* sp. 'nyererei-like'. The female offspring of the three F1 hybrid males were not significantly biased towards preferring either of the two species (16 : 27, 12 : 16 and 11 : 14, binomial tests $p = 0.072$, $p = 0.57$ and $p = 0.69$) and there was no difference in ratios between the offspring of the three males ($\chi^2 = 0.384$, d.f. $= 2$, $p = 0.82$). The experimental design of the present study did not allow us to confidently rule out that females from different broods differed in preferences, because most broods were small. However, the data rule out a general maternal effect. When restricting the analyses to broods with more than or equal to four females, four out of 36 pairwise comparisons between broods yield $p < 0.05$ with the lowest *p* being $p = 0.015$. All these are far from significant when correcting for multiple comparisons. Furthermore, while their F2 hybrid brothers show considerable colour segregation within broods, there is no indication of a strong correlation between a female's preference and the colour phenotype of her brothers (electronic supplementary material, figures S3 and S4).

### (d) There is no sign of copying of previous choice

Only 26 out of the 69 F2 hybrid females with more than or equal to five spawning decisions spawned with both species. Of those females, 21 switched back and forth between species (figure 1). This demonstrates that females do not simply copy

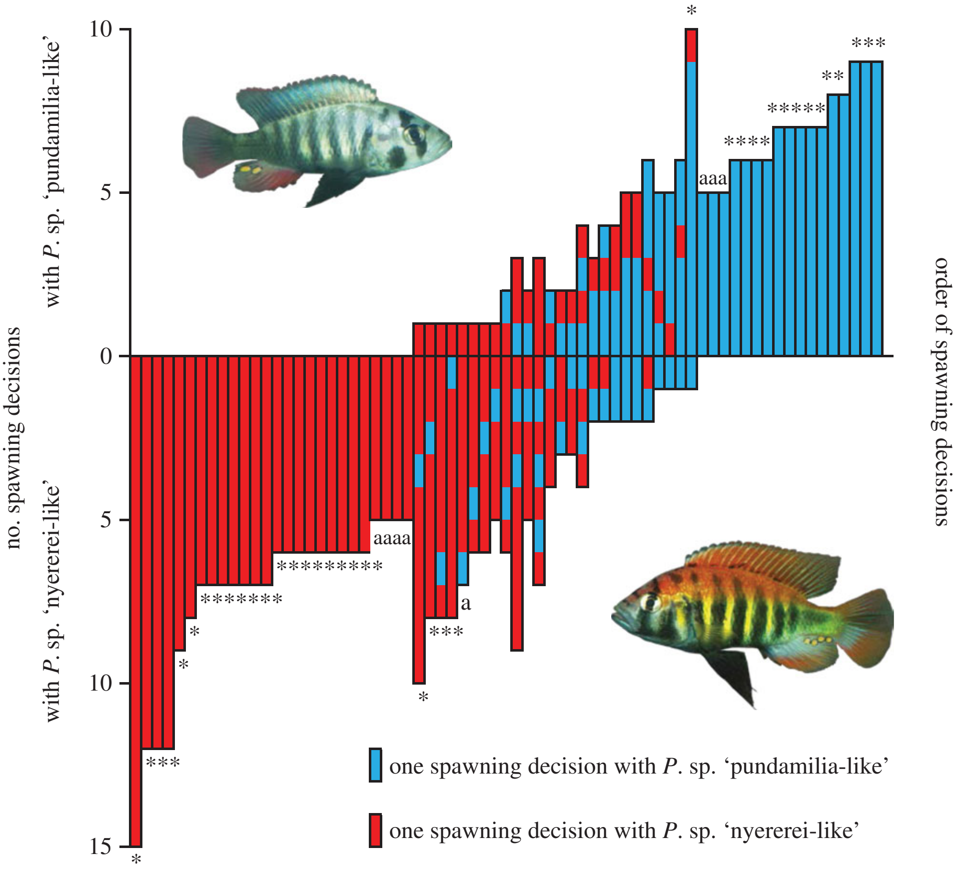

**Figure 1.** Individual spawning decisions by the 69 F2 hybrid females. Spawning decisions were determined by microsatellite DNA paternity analyses. Above the line $y = 0$ is the number of spawning decisions with *P.* sp. 'pundamilia-like', and below the line is the number of spawning decisions with *P.* sp. 'nyererei-like'. The order of spawning decisions is arranged with the first spawning on the top, and the last on the bottom with a spawning decision with *P.* sp. 'pundamilia-like', marked in blue and a spawning decision with *P.* sp. 'nyererei-like' marked in red. $*p < 0.05$, $^{a}0.05 < p < 0.1$.

their first mate choice or their most recent choice. In other words, the high repeatability of mate choice decision is unlikely to be the result of copying a previous choice.

Six of the 16 F2 hybrid females with a significant mating preference, which were enclosed with a male of the non-preferred species, did not spawn at all, and one female that did spawn, did not spawn again when reintroduced to the large choice experiment tank. The nine females that had spawned in the no-choice situation against their preference and subsequently spawned again in the choice experiment, all reverted to spawning with males of the previously preferred species (*P.* sp. 'pundamilia-like' preferring $N = 3$, *P.* sp. 'nyererei-like' preferring $N = 6$, Two tailed binomial test $p = 0.004$) which highlights the innate strength of female mate preference.

## 4. Discussion

The genetics of female mate preferences is likely to affect evolutionary processes, including speciation and hybridization between species. We report a long-term common garden study where we followed spawning decisions of F2 hybrid females between two sympatric sister species throughout a large part of their reproductive lives. Specifically, we examined if mate preferences were consistently maintained across many reproductive cycles, which included mouthbrooding and egg maturation. In addition, we also estimated spawning preferences of F1 hybrid females. Wild-type females of both species were used as a control.

Using molecular paternity testing, our experiments indicated that wild-type females mostly mated with conspecific males, although mating was not 100% assortative. This is consistent with the results of previous studies on the same population using mating experiments [30] or behavioural preference assays [9,30,42,43], and indicates that either method can be used reliably to estimate preferences. The occasional disassortative mating is also consistent with modelling based on population genomic data suggesting ongoing gene flow between the same sympatric species in nature, as well as between allopatric populations [19].

All F1 hybrid females mated with their maternal species, although a couple of them also mated with the paternal species. This bias towards the maternal species is consistent with an effect of imprinting, which had previously been demonstrated in Lake Victoria haplochromines using controlled cross-fostering experiments with mate preferences assayed with a behavioural choice test [44,45]. Our results are, however, also consistent with the possibility that genes influencing species-specific preferences were not entirely reciprocally fixed between the wild-type individuals used to breed our F1 hybrids, e.g. as a result of occasional introgression [19,26]. It is not impossible that one of the parents of our two test F1 families may have been heterozygous at a mate preference locus, and that thus some of the F1 hybrid females were homozygous.

By contrast, the experimental design limited the potential for any imprinting of species-specific preferences in F2

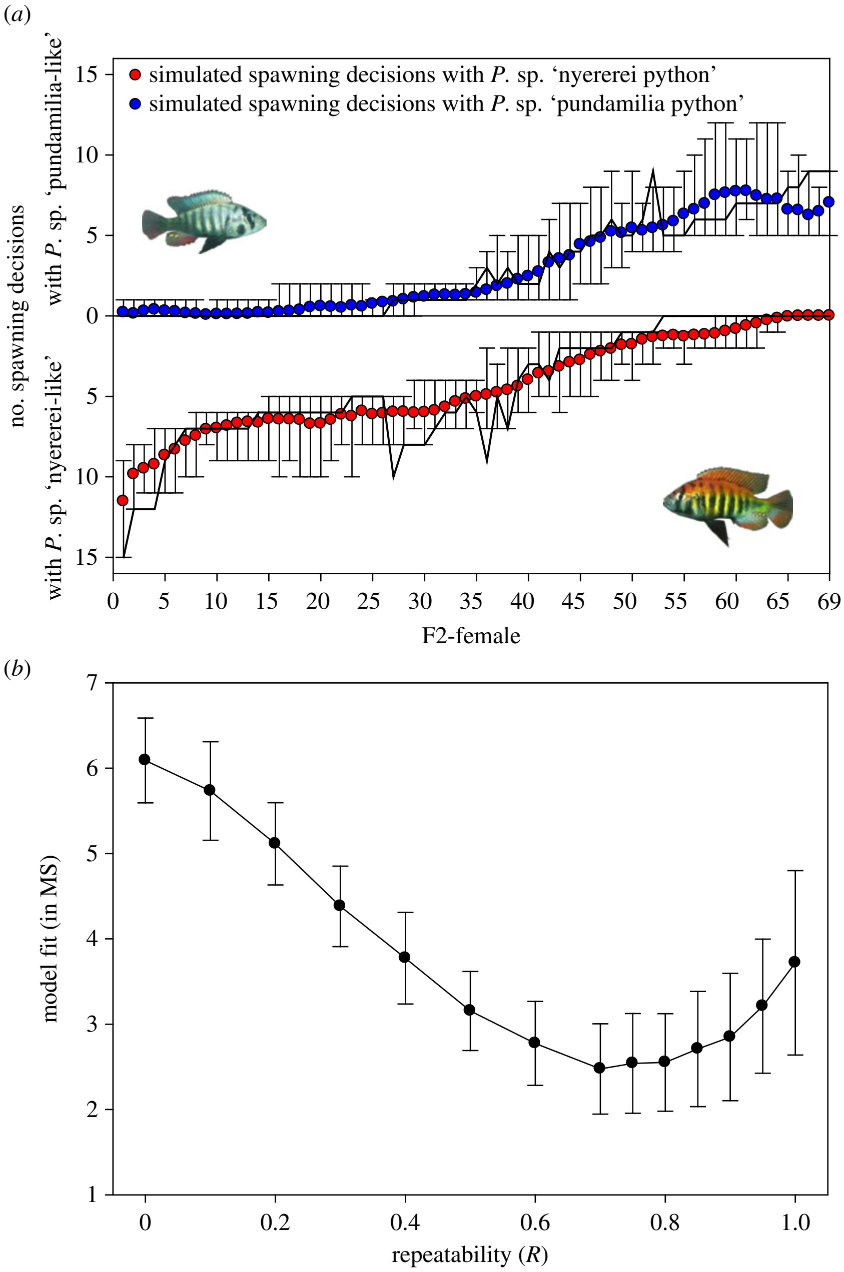

**Figure 2.** (*a*) Simulated (means and 5–95% error bars) spawning decisions of F2 hybrid females with *P.* sp. 'pundamilia-like' (blue dots), and with *P.* sp. 'nyererei-like' (red dots) based on a repeatability of an individual's spawning decision of $R = 0.7$. Observed ratio of spawning decisions is shown by the solid black lines. (*b*) The best fit of the model is with $R = 0.7$, which minimizes the mean squares (MS) between the observed and simulated spawning distribution. Lower values of $R$ produce a more random spawning pattern, while higher values of $R$ increase the consistency of a females' spawning choices above those observed, which reduced the fit of the model by inflating the MS.

hybrids, because their mothers were all F1 hybrids. Furthermore, we found that siblings in most families exhibited consistent preferences for males of different species, which is inconsistent with imprinting. Likewise, our experimental test of the preferences of females following a 'no-choice' mating with the non-preferred male species indicated that females retained their original preferences in a subsequent free choice experiment, suggesting that experience did not disrupt their innate preferences. In general, many F2 hybrid females were consistent in choosing males of a particular species, with 41 out of 59 females showing a significant preference, far more than the three expected if females had mated by chance. This clear nearly Mendelian segregation in spawning preferences in the F2 generation is consistent with previous behavioural choice tests by Haesler & Seehausen [9]. The Mendelian segregation despite incomplete genetic isolation and recombination [17,19,26] in this species pair in the wild implies that species-specific female mate choice among the *Pundamilia* sister species is influenced by relatively few major genes or genomic regions containing several tightly linked loci.

## (a) Repeatability and the heritability of mate choice

Our simulation indicated that the distribution of spawning decisions over F2 hybrid females deviated significantly from expectations if mating was random when analysed at the population level. A large excess of females showed a significant preference for males of either one of the two species. Female choice of certain type of males within a species often has low repeatability and is subject to change depending on e.g. experience, age, condition, mate copying and the environment [46–48]. In our experiment, repeatability of spawning decisions of F2 hybrid females was high (70%) and preferences did not change over time and over successive reproductive cycles of females, nor after the experience of a successful spawning event with a male of the non-preferred species. Repeatability is also often used to determine the upper-bound estimate of the broad sense heritability ($H^2$) in behavioural studies [46,47]. The results from our simulation therefore suggest that up to 70% of the variation in spawning decisions observed among the F2 hybrid females may have a heritable basis. However, the remaining 30% could simply be due to lack of a consistent preference in the class of preference heterozygote F2 hybrid females—these are expected to mate randomly [9]. Therefore, heritability may be higher than the estimated 70% [9,49]. In the experimental design, we aimed to minimize environmental variation introduced by differences in condition between males by providing a choice among eight males, four of each species in each trial. Differences in territory quality were unlikely in the standardized conditions of our experiment. Thus, we conclude that the observed among-female variation in preference is likely to be due to genetic factors.

## (b) Sexual isolation by mate choice

Behavioural reproductive isolation is of key importance to understanding the rapid evolution of genetically differentiated sympatric species [1,41,50], such as those in African cichlid fish radiations. The species pair that we studied here has been estimated to have arisen in just slightly more than 150 generations, facilitated by hybridization between the local *P. pundamilia* and migrants of *P. nyererei* from around Makobe island [19].

Theoretical work suggests that it is easier for divergent selection to overcome homogenizing gene flow if traits under divergent selection are due to relatively few genes, because the fewer genes that are responsible for a trait under divergent selection, the higher the selection coefficients for each locus [51–53]. Behavioural courtship traits involved in reproductive isolation are often, but not always, mediated by few loci with major effects, at least in the well-studied *Drosophila* [54]. The male trait (red dorsum versus blue colour) that species-assortative female mating preferences are based on in the species pair of this study [30] is probably oligogenic itself [31]. Hence, the genetic architecture of behavioural mate choice and mating traits in *Pundamilia* may facilitate speciation in the face of gene flow, perhaps in combination with other selection pressures, as might be generated by adaptation to divergent microhabitats, particularly water depths: field studies have shown that red dorsum males tend to be found in deeper water than the blue males [26].

## (c) Candidate genes for mate choice

Candidate genes relating to species-specific mate preferences are likely to include those affecting vision. Divergence has been shown in the long wavelength sensitive opsin gene (*LWS*) [26]. In the red versus blue species pair at Makobe Island, there is also divergence in the short wavelength sensitive opsin gene (*SWS2A*) but this is not currently known in the species pair of this study [26]. At Makobe Island, there is also divergence in other putative coding regions [18], some of which may be related to vision.

Many small genomic ‘islands of differentiation’ were found to differentiate *P. pundamilia* and *P. nyererei* from Makobe Island [18]. However, the Python Island species pair having recently (around 150 generations ago) re-emerged after a period of massive introgression might be expected to be divergent at fewer regions, more directly related to divergent selection pressures, which should make traits directly related to reproductive isolation easier to detect. Malinsky *et al*. [55] identified several genomic regions with high differentiation in two young ecomorphs of crater lake haplochromine cichlids (genus *Astatotilapia)* with partial assortative mating. Candidate adaptive genes in these so-called ‘genomic islands of differentiation’ included rhodopsin and other twilight-vision-associated genes. Differentiation in such ‘islands’ can resist ongoing gene flow, as shown in less than 150 year old incipient *Gasterosteus* stickleback species pairs in two Swiss lakes [56,57].

## (d) To conclude

We show in a common garden long-term mating experiment that strong female mating preferences for males of either one of two sister species are recovered in large fractions of the F2 hybrid generation. The genetic assays of mate choice in F2 hybrids between *P.* sp. ‘pundamilia-like’ and *P.* sp. ‘nyererei-like’ show high repeatability and consistency in female choice across many reproductive cycles, and we argue that the variation is influenced by the segregation of a few genes with large effects. We propose that a simple genetic basis could help facilitate stable phenotypic differentiation in sympatry in the face of gene flow.

Ethics. This work was ethically reviewed and performed under a UK Government Home Office Licence (PPL 60/3295).

Data accessibility. The complete datasets of the wild-type, F1 and F2 females, and the males used in the experiment are included in figure 1 and electronic supplementary material, figures S2 and S3 and tables S1–S4. The raw data in the electronic supplementary material, tables S1–S4 are also available from the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.q58hr [58]. The Minitab 12.1 macro to test the repeatability of mate choice is deposited at GitHub https://github.com/Ward9250/FishSpawn.

Authors’ contributions. G.F.T. and O.Se. conceived the project, O.Sv., G.F.T. and O.Se. designed the experiments, K.W. and A.S. carried out the crosses, O.Sv., K.W. and A.S. performed the experiments, O.Sv. carried out microsatellite paternity analyses and processed the data, C.v.O. wrote the simulation model, O.Sv. and C.v.O. analysed the data, O.Sv. wrote the manuscript with important contributions from C.v.O, G.F.T. and O.Se. All authors have provided critical revision of the manuscript and approved the final version.

Competing interests. We declare we have no competing interests.

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