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Research Article

Segregation of Species-Specific Male Attractiveness in F_2 Hybrid Lake Malawi Cichlid Fish

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Among the huge radiations of haplochromine cichlid fish in Lakes Malawi and Victoria, closely related species are often reproductively isolated via female mate choice although viable fertile hybrids can be produced when females are confined only with heterospecific males. We generated F_2 hybrid males from a cross between a pair of closely related sympatric cichlid fish from Lake Malawi. Laboratory mate choice experiments using microsatellite paternity analysis demonstrated that F_2 hybrid males differed significantly in their attractiveness to females of the two parental species, indicating heritable variation in traits involved in mate choice that may contribute to reproductive isolation between these species. We found no significant correlation between male mating success and any measurement of male colour pattern. A simple quantitative genetic model of reproductive isolation suggests that there may be as few as two chromosomal regions controlling species-specific attractiveness. We propose that adaptive radiation of Lake Malawi cichlids could be facilitated by the presence of genes with major effects on mate choice and reproductive isolation.

1. Introduction

The East African Great Lakes Malawi, Tanganyika, and Victoria harbour hundreds of endemic cichlid species and have served as natural laboratories for the study of speciation over the last few decades [1, 2]. The majority of these cichlids are maternal mouthbrooders, showing strongly differentiated sex roles, sexual colour dimorphism, and interspecific and geographic variation in male coloration. Patterns of female mate choice [3] and male-male competition [4] are consistent with strong sexual selection. Prezygotic isolation by direct behavioural mating preferences (sometimes based on visual cues) has been shown to be the main reproductive isolating barrier among closely related sympatric species (reviewed in [5]). Hence, sexual selection by female mate choice and male-male competition has been hypothesized to be an important diversifying force in these young species flocks [5–9].

In a well-studied sympatric species pair from Lake Victoria (*Pundamilia nyererei* and *Pundamilia pundamilia*), female mate choice has been shown to be influenced by male nuptial coloration. Females choosing amongst F_2 hybrid males prefer those that resemble males of their own species in colour [5, 10]. The differences in nuptial coloration between the two species appear to have an oligogenic basis [11]. However, there is a lack of similar investigations among other African cichlid species, and hence, it remains unknown to which extent this is a typical pattern and perhaps a causal

influence on the rapid speciation and adaptive radiation of these fishes.

Although little is known of the genetic basis of species differences in mate preferences in other African cichlid fishes, there is evidence that among Lakes Malawi and Tanganyika cichlids female preferences are important in maintaining reproductive isolation [8, 12], but the sensory basis of these preferences may be more complex. Among Lake Malawi cichlids, the *Pseudotropheus zebra* species complex (sometimes called *Maylandia* or *Metriaclima*) has been especially well studied, but evidence for the importance of different mate choice cues is mixed. In experiments with two sympatric, distinctly coloured *Pseudotropheus* species, females of both species mated assortatively using visual cues only [13]. Experiments with closely related, allopatric populations have revealed substantial assortative mating between populations with strikingly different male nuptial colour and random mating between populations with more similar coloured males [8]. However, Blais et al. [14] demonstrated assortative mating not only between geographically proximate populations with different male nuptial colouration, but also between allopatric populations of similar colour. The two species used in the present study, *Pseudotropheus emmiltos* (Stauffer, Bower, Kellogg, and McKaye) and *P. fainzilberi* Staeck, are members of the *P. zebra* species complex. They co-occur at Mphanga Rocks and Luwino Reef off the North Western coast although the distribution of *P. fainzilberi* extends to the North and South of the zone of sympatry. Behavioural reproductive isolation has been shown to be maintained under monochromatic light, but broke down when direct contact between male and female was prevented, indicating that nonvisual cues, such as olfactory signals, might facilitate reproductive isolation between these species [14, 15]. A signature of divergent selection on MHC class II genes has been demonstrated, most likely driven by different parasite communities infecting these species [16] and courting males producing significantly different sounds [17]. However, the sensory basis of assortative mating remains unclear.

The present study investigates mating preferences of nonhybrid females of these two species when given a choice among F_2 hybrid males of a pair of closely related sympatric Lake Malawi cichlid fish species (Figure 1), with all sensory cues available. This experiment could give us an indication if species differences in male attractiveness might be controlled by relatively few genes, and therefore, whether it might be tractable to try to identify such genes. We used the mate preference data to construct a simple model to make a preliminary assessment of the minimum number of genes responsible for differences among males in attractiveness to females of the two species.

2. Methods

2.1. Experimental Fish. Individuals used in this study were wild-caught or laboratory-bred fish originating from Mphanga Rocks. F_1 hybrids were produced by crossing *P. emmiltos* females with *P. fainzilberi* males and F_2 produced by in-crossing F_1 hybrids. Most F_2 males looked like F_1 hybrid males (intermediate), but a few males showed greater similarity in colour pattern to each of the parental species (Figure 1).

2.2. Mate Choice and Paternity Analyses. Preferences of wild-type females for F_2 hybrid males were simultaneously tested for both species, using three groups of four F_2 hybrid males. In the first two replicates, males were allocated randomly, but in the third replicate, to maximise phenotypic divergence, males were selected from our pool of hybrids for their visual similarity to one of the two parental species (Figure 1). Mate choice was tested using the "partial partition" design [8]. An aquarium measuring $300\,\text{cm} \times 80\,\text{cm} \times 40\,\text{cm}$ was divided into five equally sized compartments by plastic grids. Mesh size of the plastic grids was adjusted to confine males in their compartments but to allow the smaller females to pass through. Four chambers each contained one male with a clay flower pot serving as a refuge/spawning site, while one chamber was accessible to females only.

Altogether, six wild-caught and nine lab-bred *P. emmiltos* females, and ten wild-caught and 19 lab-bred *P. fainzilberi* females spawned in the experiment. Lab-bred females were raised in single-species mixed-sex groups. Each group of four males stayed in the tank for eight months, but males were interchanged between compartments at least twice during this period. Consecutively, the three replicates lasted for two years (2007-2008). The same females were used throughout the experiments, but not all females spawned in all of the three replicates. All experimental fish were marked with passive integrated transponder tags (PIT tags) and a small piece of the dorsal fin was cut off and preserved in ethanol as a DNA sample. Females carrying eggs were placed in a breeding tank until the eggs hatched, after which embryos were removed from the female's mouth, euthanized using MS-222 (tricaine methanesulfonate) and stored in 95% ethanol. Females were then released back into the experimental tank.

Where possible, four embryos (one brood contained only two embryos) from each of 100 broods, their corresponding mothers, and the 12 males were genotyped at 5–7 microsatellite loci, Ppun5, Ppun7, Ppun21 [18], Pzeb1, Pzeb3 [19], UME003 [20], and UNH130 [21]. Methods for DNA extraction and PCR reactions were as described previously [10, 14]. The amplified DNA samples were genotyped on a Beckman Coulter CEQ 8000 capillary sequencer or an ABI 3130 sequencer and sized in comparison to LIZ500(-250) (ABI) internal size standard. Genotypes and paternities were determined manually using the CEQ 8000 Series Genetic Analysing System 8.0.52 software or Peak Scanner (v. 1.0, ABI). When a female spawned with more than one male, it was regarded as one spawning decision with each father.

2.3. Visual Hybrid Index. The males of the two species have clear and discrete difference in colouration. In *P. emmiltos*, the dorsal fin and the soft parts of the caudal fin are orange, and the anal fin has a black stripe. The dorsal and caudal fins are white/blue in *P. fainzilberi*, and the dorsal fin also has a black longitudinal stripe. The underside of the body and the lower half of the head are dark grey in

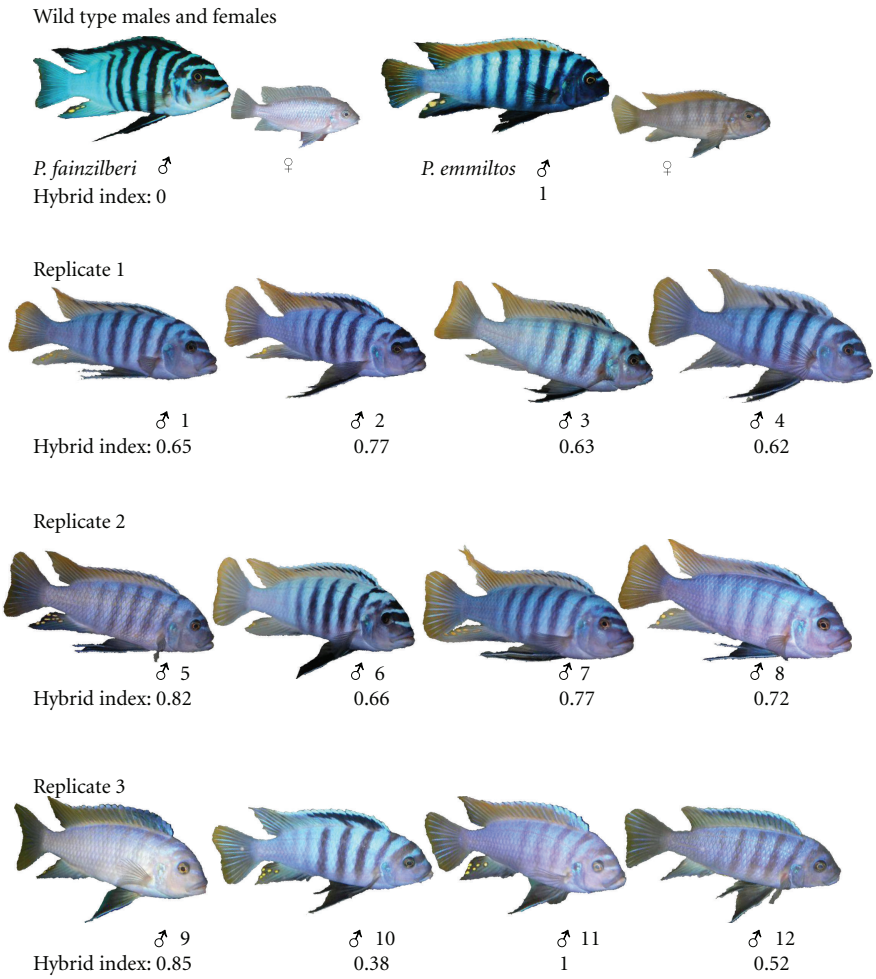

Figure 1: Wild-type males and females and second-generation hybrid males. Wild-type male and female *Pseudotropheus fainzilberi* and *P. emmiltos* from Mphanga Rocks, Lake Malawi, as well as the twelve second-generation (F_2) male hybrids used in replicates 1–3. The hybrid index was based on variation in colour of the dorsal, caudal, and anal fins. Note that the wild-type males illustrated show fully developed territorial/breeding dress, whereas the F_2 males show less well-developed colours, and vary in their degree of expression. Photos by Markos Alexandrou, Alan Smith, and Katie Woodhouse

displaying male *P. emmiltos*, whereas these are white/light blue in *P. fainzilberi*. In addition to these characters, there are more subtle differences in, for example, body shape. All males were observed during the experiment. After each replicate was completed, photographs of all males were taken on at least two days. There was little variation in the head colours of the hybrids, and it seemed to be very influenced by motivational state and could not be clearly distinguished using our photographs, so this measure had to be omitted. Thus, we calculated a hybrid colour index based on variation in colour of the dorsal, caudal, and anal fins. Giving these three traits equal weight, a continuous visual hybrid index was calculated (by OSv), where 0 = state in wild-type *P. fainzilberi* and 1 = state in wild-type *P. emmiltos*. The traits were determined using photographs standardised against mean values for males of each species (*P. emmiltos*: 4 wild-caught captive males and 10 males photographed in the lake, *P. fainzilberi*: 4 wild-caught captive males and 3 males photographed in the lake). The dorsal and caudal fin measurements were calculated from the specifically coloured areas (e.g., the proportion of orange colouration in the caudal fin of a F_2 hybrid male was divided by the proportion of orange colouration averaged for the 14 *P. emmiltos* males), and the anal fin measurement was based on the number of the first six spines being black.

2.4. Testing Spawning Decisions. A randomisation procedure was used to test the null hypothesis that males were equally attractive to *P. fainzilberi* and *P. emmiltos* females. We used a Monte Carlo method with 1000 simulations per replicate to simulate the spawning decisions of a total of 44 females (i.e., 15 *P. emmiltos* and 29 *P. fainzilberi* females) with 12 F_2 hybrid males (in three replicates). We calculated the probability of finding a combination of spawning decisions that is more uneven than the one observed, assuming that all males were equally attractive. The expected number of spawnings with male Mi by *P. fainzilberi* (Pf) females was therefore, calculated as $N(\text{Mi} \times \text{Pf}) = N_{\text{Mi}}N_{\text{Pf}}/(N_{\text{Pf}} + N_{\text{Pe}})$,

where N_{Mi} is the total number of spawnings received by male (Mi), N_{Pf} is the total number of spawnings summed across all *P. fainzilberi* (Pf) females in the replicate, and N_{Pe} is the total number of spawnings by all *P. emmiltos* females in the replicate. The expected number of spawnings of *P. emmiltos* (Pe) females with male Mi was calculated in the same manner. We randomised the females, allocating the observed total number of spawnings to each male, and then calculated the absolute deviation between the mean expected values and each of the simulations (Δsim). This simulated distribution of deviations was then used to statistically test the deviation between the observed and the expected values of the numbers of spawnings for each male (Δobs). This procedure tests whether the relative preference for a particular male differs significantly between the two species of females. The macro for this procedure was written in Minitab 12.1 and is available from the authors upon request. In addition, we tested the spawning decisions for each male separately using a binomial equation [22]. The probability of spawning of a Pe and Pf female was equal to their relative proportions in the replicate.

To test for associations between female preference and visual similarity of F_2 males to conspecific males, we used a general linear model (GLM) ANCOVA with males nested within replicate (random factor), the male's hybrid colour index as covariate, and with the observed deviation from the expected number of spawnings (Δobs) as response variable. Minitab 12.1 was used for the statistical calculations.

2.5. Estimating the Number of Reproductive Isolation Genes. We constructed a simple quantitative genetic model to estimate the minimum number of genes/chromosomal regions that determine species-specific male attractiveness. In the model, we assumed that the results were purely based on genetic differences between the males and that k genes = $1, 2, \ldots, 5$ have an equal effect on female mate preference. The model, furthermore, assumes that males become unattractive to females of one species only when they are homozygous for k heterospecific alleles. The proportion of such fully restored F_2 individuals (Prop{restored}) decreases as a function of the number of reproductive isolation genes, Prop{restored} = $2/(2^{2k})$, where k equals the number of genes. We then calculated the binomial probability of finding the observed pattern of mate-choice decisions (i.e., 2 out of 12 males being significantly disfavoured by females of one species), for different values of k.

3. Results

We successfully determined paternity for 395 out of 398 embryos. By comparing the simulated with the observed data we rejected the hypothesis that all males are equally attractive to females of the two species in replicate 1 (P = .011) and replicate 2 (P = .001), where males were allocated randomly, but not in replicate 3 (P = .091) (Figures 2(a)–2(c)), where we had attempted to maximise phenotypic divergence between the four males. Male 1 in replicate 1 and male 6 in replicate 2 received no spawnings from female *P. fainzilberi*, but were successful with *P. emmiltos* females (Figures 2(a) and 2(b)). The probability of finding such extreme bias in number of spawnings was exceedingly small (Binomial test for male 1 replicate 1: $P = 6.73 \times 10^{-5}$, and for male 6 replicate 2: $P = 3.74 \times 10^{-5}$). Thus, two out of 12 F_2 hybrid males were significantly avoided as mates by females of one of the species. The variation in Δobs was not explained by the replicate nor by the visual hybrid index (GLM nested ANCOVA: random factor replicate $F_{2,6} = 1.20$, P = .36, covariate hybrid index $F_{3,6} = 0.82$, P = .53).

Under the assumptions of our simple quantitative genetic model, this result is consistent with female preference being based on male traits coded by a minimum of two chromosomal regions carrying major genes (Figure 3). If there was only a single chromosomal region affecting species-specific male attractiveness, we would have expected more F_2 males to be avoided by females of one of the species. On the other hand, if there were more than four chromosomal regions carrying genes of major effect on species-specific male attractiveness, the model predicts we would be unlikely to find any males unattractive to females of either species.

4. Discussion

The present study shows that male F_2 hybrid *P. emmiltos* × *P. fainzilberi* differ in their attractiveness towards females of the two parental species, indicating heritable variation in traits involved in mate choice and thus reproductive isolation between these species. Although the conclusions are based on 153 spawning decisions of 44 females choosing between only 12 males, the bias in female preference is statistically and biologically highly significant. The observation that 2 out of 12 males did not attract a single spawning of the 29 *P. fainzilberi* females implies there might be a simple biological mechanism responsible for restoring species-specific attractiveness.

Here, we argue that if we assume that species-specific attraction has a genetic basis, the genes responsible can be found in a minimum of two chromosomal regions. Likewise, F_2 hybrid males from crossing the Lake Victoria cichlid fish *Pundamilia pundamilia* and *P. nyererei* varied in attractiveness to wild-type females [10]. Females of each Victorian cichlid species preferred F_2 hybrid males that had a body colour similar to those of their conspecific nonhybrid males. In contrast, we were unable to find an association between female preference and our measures of male colour variation in the Malawi cichlids that we studied. Previous studies have suggested that visual cues are inadequate to maintain reproductive isolation among *P. emmiltos* and *P. fainzilberi* and that olfactory signals may be required [15], possibly associated with the known significant differences among these species in MHC allele frequencies [16]. Males of these species also produce significantly different courtship vocalisations [17]. Furthermore, the females of the two parental species differ slightly in colour and body shape (and perhaps in other signals). Individual variation in male mating preferences for different (conspecific) female colour morphs has been documented in a closely related colour-polymorphic Malawian cichlid fish [23, 24]. Variation in species-specific attractiveness of F_2 males used in our study

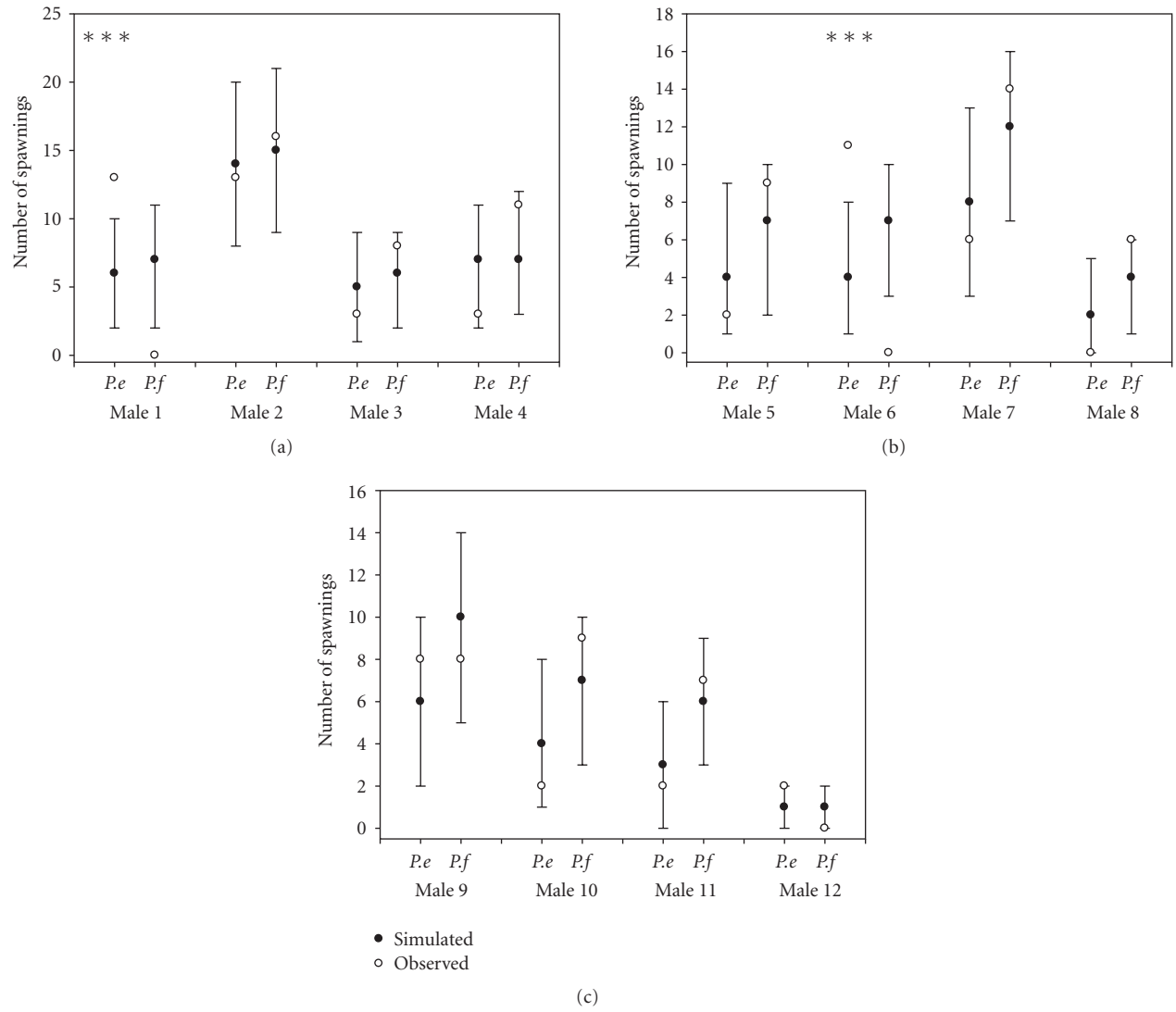

Figure 2: Spawning decisions of wild-type females spawning with hybrid males. The distribution of observed (open circles) and simulated (filled circles) spawning decisions of wild-type *P. emmiltos* and *P. fainzilberi* females spawning with four F_2 hybrid males in replicates 1, 2, and 3. The probability of finding the observed number of spawnings for each male was calculated using a binomial equation. (***indicates $P < .001$). The bars show the 5–95% confidence limits of the simulated spawning decisions.

may have been caused by differences in nonvisual signals, by segregation of male preferences for females of the different parental types, or by hitherto not quantified visual signal variation.

Our model suggests that the observed nonrandom component of mating may have been based on a small number of chromosomal regions with major effect. Of course, our model is simplistic and differs from previously developed quantitative genetic models (e.g., [25]): it assumes that (1) all observed variation was genetic, (2) genes responsible for reproductive isolation all have similar effect size, and (3) males are avoided as mates only when they are homozygous for the heterospecific alleles at these loci. This latter assumption is consistent with laboratory mate choice experiments showing that females of this species pair do not discriminate against F_1 hybrid males; that is, males that are likely to be heterozygous for alleles relating to traits preferred by conspecific females [26]. Our approach of using a simple model with a minimum number of assumptions makes heuristic sense and is also consistent with the assumptions and inferences made for other traits in cichlids. For example, major gene effects have also been suggested to affect jaw and tooth shapes [27], colour differences [11, 28–30] female [31] and male mate preferences [30], and female behavioural dominance [32]. The fact that this species pair is significantly diverged for the MHC class II loci [16] also makes the MHC a possible candidate for reproductive isolation. In the model we assumed that the presence of a single conspecific allele is sufficient to restore female preference. This could work if the olfactory signal produced by one conspecific allele in a heterozygote suffices to restore recognition, while the absence of any conspecific MHC alleles results in mate rejection.

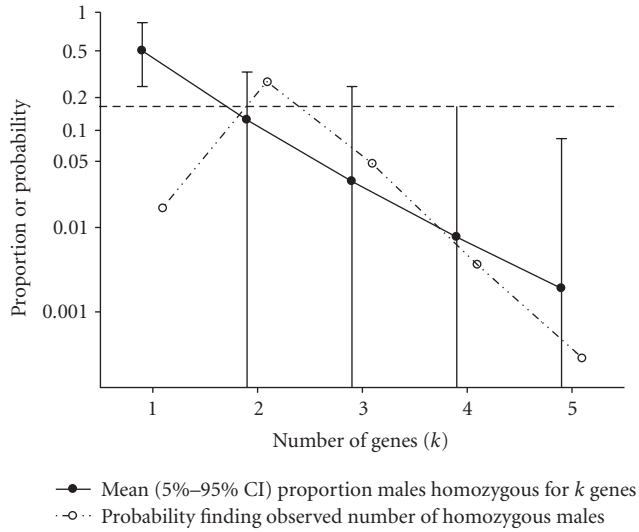

Figure 3: Result from the quantitative genetic model. Results from the quantitative genetic model and observations. Mean (±5–95% CI) expected proportion of F_2 males that are completely homozygous for the reproductive isolation genes (k) of one of the species (solid symbols and line). The open symbols and broken line give the probability of finding the observed number of F_2 males with such genotype as a function of k. The horizontal dashed line at $y = 0.167$ shows the observed proportion of males (2 out of 12) that were preferred by females of a single species only, and which are assumed to be homozygous for the reproductive isolation genes. The number of reproductive isolation genes that is most consistent with the model equals $k = 2$, while $k = 1$ and $k \geq 5$ are rejected by this model.

Major gene effects have also been identified in key traits in adaptive radiations of other taxa, such as body armour in sticklebacks [33] and in beak shape in Darwin's finches [34]. Our experimental results in combination with the recent technological breakthrough of sequencing of restriction site associated DNA (RAD) tags (see [35]) would allow us to fine map the genetic basis of reproductive isolation genes (and hence, speciation genes) by identifying recombinant breakpoints in F_2 individuals with restored phenotype. Furthermore, it has been suggested that major gene effects are often key to rapid adaptive change, as a result of widespread fluctuation of environmental parameters leading to variable selection pressures [36], which are likely to have occurred in Lake Malawi [37–39].

In conclusion, the present study shows that male F_2 hybrid *P. emmiltos* × *P. fainzilberi* vary in their attractiveness towards females of the two parental species. This preliminary finding suggests that these species may be genetically differentiated for heritable variation in traits involved in mate choice although we cannot rule out nongenetic effects. Furthermore, we were unable to find an association between female preference and our measures of male colour variation in our hybrids. We propose that the adaptive radiation of haplochromine cichlids in Lake Malawi and elsewhere could be facilitated by the presence of genes with major effects on behavioural reproductive isolation, as well as perhaps other traits.

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