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Sympatry and parapatry among rocky reef cichlids of Lake Victoria explained by female mating preferences

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Abstract

Work on the Lake Victoria cichlids *Pundamilia nyererei* (red dorsum males, deeper water), *Pundamilia pundamilia* (blue males, shallower water) and related species pairs has provided insights into processes of speciation. Here, we investigate the female mating behaviour of 5 *Pundamilia* species and 4 of their F1 hybrids through mate choice trials and paternity testing. Complete assortative mating was observed among all sympatric species. Parapatric species with similar depth habitat distributions interbred whereas other parapatric and allopatric species showed complete assortative mating. F1 hybrids mated exclusively with species accepted by females of the parental species. The existence of complete assortative mating among some currently allopatric species suggests that pre-existing mating barriers could be sufficient to explain current patterns of co-existence, although, of course, many other factors may be involved. Regardless of the mechanism, mating preferences may influence species distribution in potentially hybridizing taxa, such as in the adaptive radiation of cichlid fish. We suggest that this at least partly explains why some species fail to establish breeding populations in locations where they are occasionally recorded. Our results support the notion that the mating preferences of potentially cross-breeding species ought to be included in coexistence theory.

Keywords: adaptive radiation, coexistence theory, hybridization, mate choice, range expansion, reproductive isolation

Introduction

Why do some species coexist in sympatry, whereas others occur only in allopatry or parapatry (contiguously: Bull, 1991)? To answer this question, speciation theory focuses on the evolution of barriers to gene flow starting from a single interbreeding species, whereas ecological coexistence theory generally assumes species to be completely reproductively isolated and considers factors such as habitat and diet partitioning (Irwin & Schluter, 2022; Mittelbach & McGill, 2019). In terms of speciation theory, strongly reproductively isolated species can be expected to co-occur in sympatry or allopatry/parapatry, whereas more weakly isolated populations are only able to occur with a measure of geographic separation. Theoretically, this may be due to the homogenization of gene pools of less strongly assortative populations through interbreeding in sympatry (Kirkpatrick & Barton, 1997; Mallet, 2008; Sexton et al., 2009; Templeton, 1981). Such differential fusion of populations with similar mating and preference traits is sometimes called the "Templeton effect" (Coyne & Orr, 2004; Templeton, 1981). Furthermore, where hybrids have reduced fitness, selection may strengthen assortative mating, through the process of "reinforcement" of mating preferences (Coyne & Orr, 2004; Hopkins & Rausher, 2012; Nosil, 2012; Servedio & Noor, 2003). Even

if taxa are genetically completely incompatible, reproductive interference (e.g., production of inviable hybrids, sexual and territorial competition, etc.) may be costly, even to the extent of making it impossible for species to coexist (Groning & Hochkirch, 2008; Kyogoku, 2020). This may also drive the divergence of mating traits, resulting in a pattern where sympatric species have more strongly developed assortative mating than parapatric and allopatric species (Coyne & Orr, 2004; Noor, 1999). Theoretical modelling integrating these approaches, considering ecologically differentiated species with incomplete reproductive isolation suggests that, unless hybrid survival is low, assortative mating has to be very strong to allow co-existence (Irwin, 2020; Irwin & Schluter, 2022). Such interactions may prevent species from becoming established within the range of neighbouring, locally more common species, leading to parapatric or allopatric distributions (Mallet, 2008; Rhymer & Simberloff, 1996; Seehausen & Schluter, 2004).

Lake Victoria hosts a large number of closely related haplochromine cichlid fish species, many co-occurring in sympatry, but many also have restricted and non-overlapping geographic ranges (Seehausen, 1996). Closely related species are often little differentiated in morphology but strongly divergent in male nuptial colour patterns (Seehausen, 1996).

Hybridization and introgression are not uncommon (Keller et al., 2013; Konijnendijk et al., 2011; Meier et al., 2017a, 2017b; Seehausen, 1996; Seehausen et al., 1998a) and have been important features in the evolution of the species flock from its onset (Meier et al., 2017a). The species flock has evolved over the last 16,000 years (Johnson et al., 2000; Seehausen, 2006) and it is unlikely that traits such as nuptial colouration and preferences have evolved through fixation of new mutations in this short time but instead, are likely to originate from standing genetic variation with relevant gene variants much older than the species themselves (Meier et al., 2017a; Meier et al., 2023). Many closely related sympatric Lake Victoria species (some species pairs can be as young as 150 generations; Meier et al., 2017b) produce fit offspring in the laboratory (Stelkens et al., 2009; Svensson et al., 2017), but mate largely assortatively in nature and in the laboratory (Seehausen et al., 1998b; Selz et al., 2014; Svensson et al., 2017). Because this not only affects speciation but also the outcome of secondary contact after range expansions too, it opens the possibility that species distributions are influenced by mate choice traits and behaviour, in addition to other known drivers of species coexistence (Irwin & Schluter, 2022). In Lake Victoria, there is a strong correlation between ambient light, speciation, and species richness (Seehausen, 2009; Seehausen et al., 1997, 2008). In the sympatric red dorsum vs. blue species pairs *Pundamilia nyererei*—*Pundamilia pundamilia* and *Pundamilia* sp. "nyererei-like"—*Pundamilia* sp. "pundamilia-like," it has been shown that females use male nuptial colour signals both in intra- and inter-species mate choice whereas males have no assortative mating preferences (Maan et al., 2004; Selz et al., 2014).

Aims and predictions

The aim of the present study was to test if the mating preferences of five Lake Victoria cichlid species were predicted by geographical species distribution patterns of sympatry, parapatry, and allopatry or by similarities in male nuptial colouration. Two parapatric species pairs are not known to maintain breeding populations in sympatry: *P. pundamilia*—*Pundamilia* sp. "red head" and *P. nyererei*—*Pundamilia igneopinnis*. We refer to these as pairs of "complementary" species due to their potential ecological interchangeability. We therefore predicted that these lack strong behavioural reproductive isolation. Other species co-occur in some parts of their geographical range or in their entire range and because they are able to co-occur, we predicted these to show strong assortative mating. We predicted species from the major male nuptial colour pattern types "blue" (*P. pundamilia*, *Pundamilia azurea*), "red dorsum" (*P. nyererei*, *P. igneopinnis*), and "red chest" (*Pundamilia* sp. "red head") (Figure 1) to spawn assortatively, whereas the level of assortative mating between amelanic and melanic forms within "blue" and "red dorsum" (Figure 1) were uncertain. An additional aim was to test if preferences were inherited in F1 hybrid females. We predicted that F1 hybrid females inherited preferences and spawned as a combination of both parental species preferences with or without the presence of directional dominance. Alternatively, assortative mating might break down in regard to a third species. For example, if sympatric species had evolved avoidance of certain heterospecific traits, such as the disdain of sworded males in the swordless swordtail *Xiphophorus birchmanni* (Wong and Rosenthal, 2006), sympatric F1 hybrid females may even avoid males of one or both of the parental species.

Methods

The experiment was carried out over 2 years, from August 2006 to July 2008, in the aquarium facility at the University of Hull.

Study species

The five species originate from south-eastern Lake Victoria (Tanzania) around islands in the Speke Gulf, not further apart than 50 km (Figure 1). Their phylogenetic relationships are complicated and characterized by lineage fusion through admixture and lineage fission through speciation (Meier et al., 2023). They are maternal mouthbrooders with similar ecologies, inhabiting rocky shores and reefs and feeding largely on plankton and benthic invertebrates (Bouton et al., 1997; Maan et al., 2008; Seehausen, 1996; Seehausen et al., 1998a). Females have similar cryptic colouration whereas the larger males have the bright nuptial colouration of either one of three patterns that are widespread among Lake Victoria cichlid species and referred to as "blue," "red dorsum," or "red chest" (Seehausen & van Alphen, 1999; Seehausen et al., 1998a), each of which exists also in melanic forms where the underlying colour pattern can be largely disguised (Figure 1). Among rocky shore cichlids, these different colour pattern groups are associated with different habitats: "blue" and "red chest" males breed in shallow water over gently sloping substrate, "red dorsum" males breed in similar habitats but deeper, and melanic forms breed even deeper or at steeply dropping rock faces (Seehausen, 1996). Whereas females of the first group forage at the same level as territorial males, feeding on benthic macroinvertebrates, in the other two groups, they forage in the water column above territorial males, feeding on plankton (Seehausen, 1996). Our five species can be divided into three groups, substantial breeding populations of which can co-occur with species of other groups, but not with members of the same group: (i) *Pundamilia pundamilia* and *Pundamilia* sp. "red head" tend to live in shallow water, (ii) *P. nyererei* and *P. igneopinnis* tend to occur in deeper water, and (iii) *P. azurea* in yet deeper water, although all these depth ranges overlap to an extent and vary between islands. They belong to a complex of more than 20 congeneric species (Seehausen, 1996; Seehausen et al., 1998a). Male nuptial colour pattern phenotypes, species distribution and specific source populations used in our experiments are shown in Figure 1 and Table 1. Further details are given in Supplementary Information S1.

Wild-type fish

The experiment started in 2006. We used first-generation lab-bred fish bred from parents caught in Lake Victoria in 2003, except *P. igneopinnis* and *Pundamilia* sp. "red head" which were from laboratory strains established from fish caught in 1993, i.e., the experimental fish were approximately three to five lab-bred generations. Fry were removed from the mouth of their mothers when they could swim free and feed independently, and raised in large mixed-sex stock tanks until sexual maturity (55 females and 43 males). There was therefore scope for imprinting on their mothers (Svensson et al., 2017; Verzijden & ten Cate, 2007). Each female is represented by one bar in Figure 2A.

F1 interspecies crosses

We chose four species pairs based on their distributions and nuptial colour types. *Pundamilia pundamilia* ("blue") and *P.*

location number	polymorphic	P. sp. 'nyererei-like'	P. sp. 'pundamilia-like'	*P. nyererei*	*P.pundamilia*	P. sp. 'pink anal'	*P. igneopinnis*	P. sp.'red anal'	*P. azurea*	*P. sp.'red-head'*	location name
1					X						Nansio Island
2					X						Bwiru Island
3					X						Namatembi Island
4										X	**Zue Island**
5					X,X						Miandere Island
6									X	X	Mabibi Islands
7				X	X				X	*	**Ruti Island**
8				*	X				X	X	Vesi Islands
9					X						Bihiru Island
10					X		X	+			Ndurwa Point
11				X	X	X					**Makobe Island**
12				(X)	X	X	X	+	*		**Igombe Island**
13				X	X						Senga Point
14					X						Mponze Point
15				X	X	X					Juma Island
16					X	X					Juma Island
17				*	X		*				Bwiru Point
18					X	X					Wakahangara Point
19					X	X					Amranda Point
20		X	X								Karomo Bay
21				X	X						Gabalema Island
22		X	X								Hippo Island
23		X	X								Kissenda Island
24				X	X						Saa Nane Island
25				X	X						Anchor Island
26				X	X						Nyegezi Rocks
27		X	X								Ngoma Point
28				X	X						Kilimo Island
29		X	X								Nyamatala Island
30		X	X								Python Island
31	X										Shadi Rocks
32	X										Mashoro Bay
33	X										Matumbi Island
34	X										Luanso Island
35	X										small Marumbi Island
36	X										large Marumbi Island
37	X										Buyago Rocks 2
38	X										Buyago Rocks 1

P. azurea, Ruti Isand

P. nyererei, Makobe Island

P. igneopinnis, Igombe Island

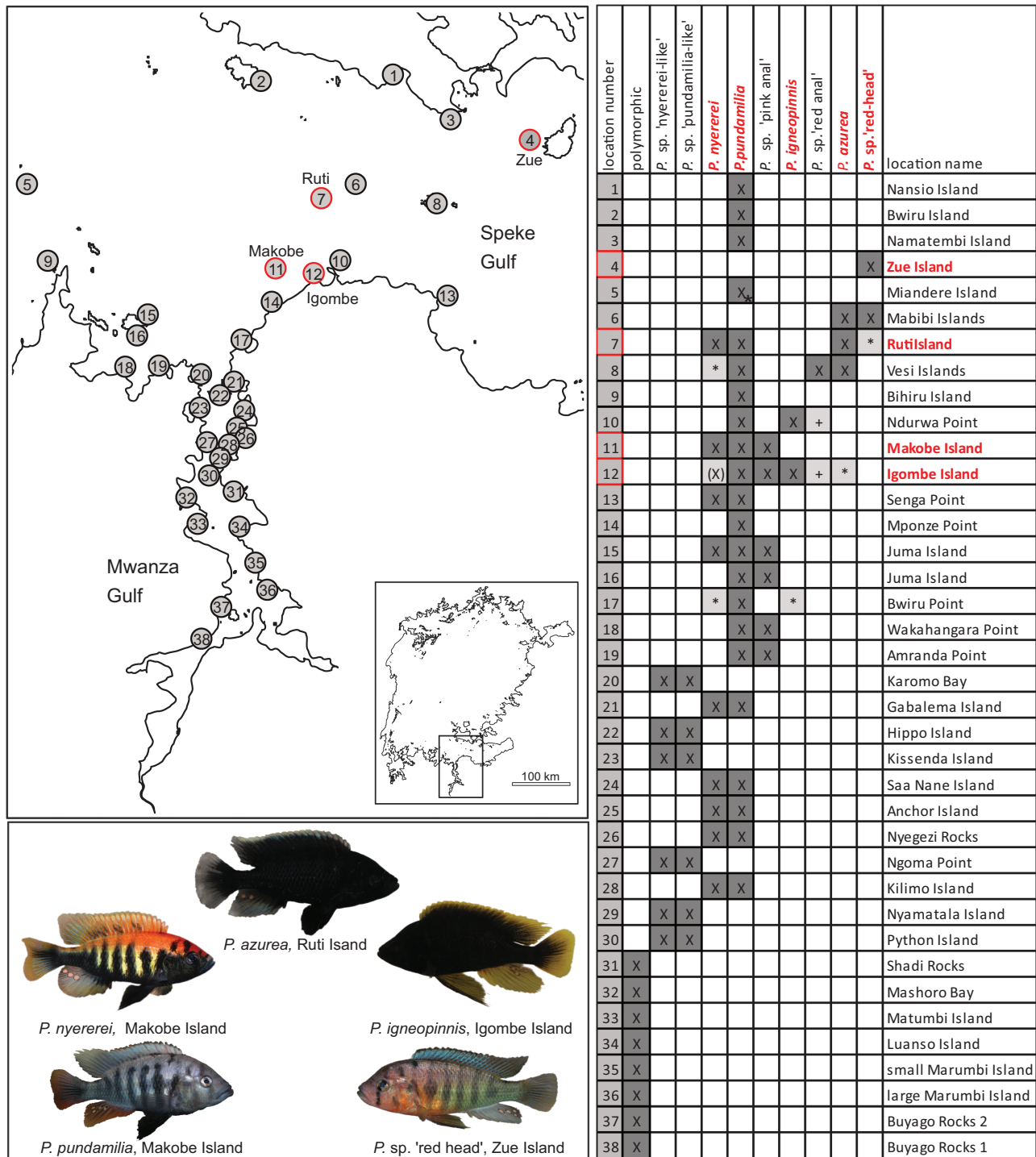*P. pundamilia*, Makobe Island

P. sp. 'red head', Zue Island

Figure 1. Mwanza bay and Speke Gulf in southeastern lake Victoria, Tanzania, with distributions and male nuptial colourations of the five *Pundamilia* spp. of the present study which represent each major male nuptial colour type typical of Lake Victoria cichlids: "blue" (*P. pundamilia*, *P. azurea*), "red dorsum" (*P. nyererei*, *P. igneopinnis*), and "red chest" (*Pundamilia* sp. "red head"), and melanic forms within "blue" (*P. azurea*) and "red dorsum" (*P. igneopinnis*) (Seehausen & van Alphen, 1999; Seehausen et al., 1998a). Highlighted in red are the species and the locations of the populations used in the present study. In the right panel are also species with similar nuptial colourations (see Supplementary Information S1) as well as polymorphic populations with a continuum between "blue" and "red dorsum." Similarities: *Pundamilia* sp. "nyererei-like"—*P. nyererei*, *Pundamilia* sp. "pundamilia-like"—*P. pundamilia*, *Pundamilia* sp. "red anal"—*P. igneopinnis*, and *Pundamilia* sp. "pink anal"—*P. azurea*. The species' distributions are marked with X and deviations are marked as follows: (X) occasionally in large numbers but no breeding populations (Seehausen, unpublished; Seehausen, 1996), *occasional occurrence of few individuals (*P. azurea,* Igombe Island, one individual 1992 (Seehausen et al., 1998a); *P. igneopinnis*, Bwiru point, one individual (Seehausen, unpublished); *P. nyererei*, Bwiru point and Vesi Island, few individuals (Seehausen, unpublished); *Pundamilia* "red head," Ruti Island, one individual (Feller et al., 2020)) and + identical nuptial colouration as *Pundamilia* sp. "red anal" but similar body shape as *P. igneopinnis*, four males (Seehausen, 1996). Depth ranges are given in Table 1. Further species information is given in Supplementary Information S1. This figure is modified from Feller at al. (2020) and data are taken from Seehausen (1996) and Seehausen et al. (1998a). Photos: Katie Woodhouse.

Table 1. Pairwise comparison of coexistence, depth distribution, and assortative matings among five Lake Victoria *Pundamilia* species

Female\Male	*P. azurea*, Ruti Island	*P. igneopinnis*, Igombe Island	*P. nyererei*, Makobe Island	*P. pundamilia*, Makobe Island	*Pundamilia* sp. "red head," Zue Island
P. azurea, Ruti Island		Sympatric* Deep vs. intermediate **Assortative (1.00)**	Sympatric Deep vs. intermediate **Assortative (1.00)**	Sympatric Deep vs. shallow **Assortative (1.00)**	Sympatric* Deep vs. shallow **Assortative (1.00)**
P. igneopinnis, Igombe Island	Sympatric* Intermediate vs. deep **Assortative (1.00)**		Parapatric Intermediate vs. intermediate **Not assortative (0.54)**	Sympatric Intermediate vs. shallow **Assortative (1.00)**	Allopatric Intermediate vs. shallow **Assortative (1.00)**
P. nyererei, Makobe Island	Sympatric* Intermediate vs. deep **Assortative (1.00)**	Parapatric Intermediate vs. intermediate **Partly assortative (0.91)**		Sympatric Intermediate vs. shallow **Assortative (1.00)**	Parapatric Intermediate vs. shallow **Assortative (1.00)**
P. pundamilia, Makobe Island	Sympatric* Shallow vs. deep **Assortative (1.00)**	Sympatric* Shallow vs. intermediate **Assortative (1.00)**	Sympatric Shallow vs. intermediate **Assortative (1.00)**		Parapatric Shallow vs. shallow **Partly assortative (0.77)**
Pundamilia sp. "red head," Zue Island	Sympatric* Shallow vs. deep **Assortative (1.00)**	Allopatric Shallow vs. intermediate **Assortative (1.00)**	Parapatric Shallow vs. intermediate **Assortative (1.00)**	Parapatric Shallow vs. shallow **Partly assortative (0.94)**	

Note. Females were allowed to choose between males of all five species in a simultaneous mate choice experiment. The proportion of spawnings with conspecifics is shown within brackets. Assortative = all spawnings were with conspecifics. Partly assortative = females spawned with both species. However, significantly more spawnings were with conspecifics than with heterospecifics. Not assortative = no significant difference in the number of spawnings with conspecifics and heterospecifics.
*The sympatric populations are at different islands from those used in the present trials, or (for *P. azurea* vs. *P. igneopinnis*) similar species. Nuptial colour patterns are shown in Figure 1 and described in Supplementary Material S1.

nyererei ("red dorsum") are part of the sympatric blue vs. red dorsum species pair model system. We took both from Makobe Island. The two parapatric complementary species pairs *P. pundamilia* ("blue")—*Pundamilia* sp. "red head" ("red chest") from Makobe and Zue Island, respectively, and *P. igneopinnis* ("red dorsum" melanic)—*P. nyererei* from Igombe and Makobe Island were crossed. The nuptial colour patterns of the latter pair are of the "red dorsum" type but in dominant/courting *P. igneopinnis* this is largely covered with melanophores, the orange being confined to the fin margins. *Pundamilia azurea* ("blue" melanic) from Ruti Island was crossed with *P. nyererei* from Makobe Island as a second sympatric cross (*P. nyererei* is sympatric with *P. azurea* at Ruti Island, Figure 1) and a second blue (or blue-melanic) vs. red dorsum cross. These crosses covered two crosses of sympatric species (albeit in one case the populations tested were not sympatric), two parapatric crosses, two red dorsum vs. blue crosses, one red chest vs. blue cross, one red dorsum vs. red dorsum cross, and two melanic vs. non-melanic crosses. To produce the crosses, three to five females were put in an aquarium together with a male of the other species. Lake Malawi cichlids were used as dither fish to reduce shyness and aggression. To avoid filial cannibalism of small clutches (Mrowka, 1987), mouthbrooding females were stripped from eggs at the eye stage or newly hatched fry and the offspring were raised artificially. Hence, there was less scope for imprinting compared to earlier studies (Svensson et al., 2017; Verzijden & ten Cate, 2007) and to the wild-type females of the present study. The juveniles were raised in 180 × 45 × 33 cm mixed-sex stock tanks until sexual maturity.

We obtained crosses in both directions for all four species pairs. However, in the *P. nyererei* × *P. azurea* and *P.* *pundamilia* × *Pundamilia* sp. "red head" pairs, females from one of the directions were produced late and reached sexual maturity after the experiment ended. The hybrid females used in the experiment were either from one female and one male (*P. nyererei* × *P. azurea*, N = 9; *P. nyererei* × *P. pundamilia*, N = 2; and *P. pundamilia* × *P. nyererei*, N = 4) or two females and one male (*P. pundamilia* × *Pundamilia* sp. "red head," N = 20; *P. igneopinnis* × *P. nyererei*, N = 14; and *P. nyererei* × *P. igneopinnis*, N = 10). Each F1 female is represented by one bar in Figure 2B. The species of the dams are written first. We will refer to *P. igneopinnis* × *P. nyererei* and *P. nyererei* × *P. pundamilia* when the direction of the cross is not relevant.

The F1 hybrid females were used in the experimental set-up, whereas a subset of the F1 hybrid males were photographed and euthanized using MS-222 (tricaine methanesulfonate).

Experimental setup

A 240 × 80 × 40 cm aquarium was divided by grids into ten male compartments with a flowerpot as a standardized spawning site in each. This "partial partition" design allowed females to move freely whereas the larger males were confined to their compartments (Supplementary Figure S1). We used standard daylight full light spectrum aquarium T5 fluorescent tubes to enable nuptial colours to be visible for all females. The combination of ten males, two from each species, was changed every second month the first year when most clutches were spawned and thereafter every 3–5 months when we collected the remaining clutches (total number of males used: 9 *P. azurea*, 10 *P. igneopinnis*, 8 *P. nyererei*, 8 *P. pundamilia*, and 8 *Pundamilia* sp. "red head"; Supplementary Tables S1 and S2). All experimental fish were marked with Passive Integrated Transponder (PIT) tags and a clip from
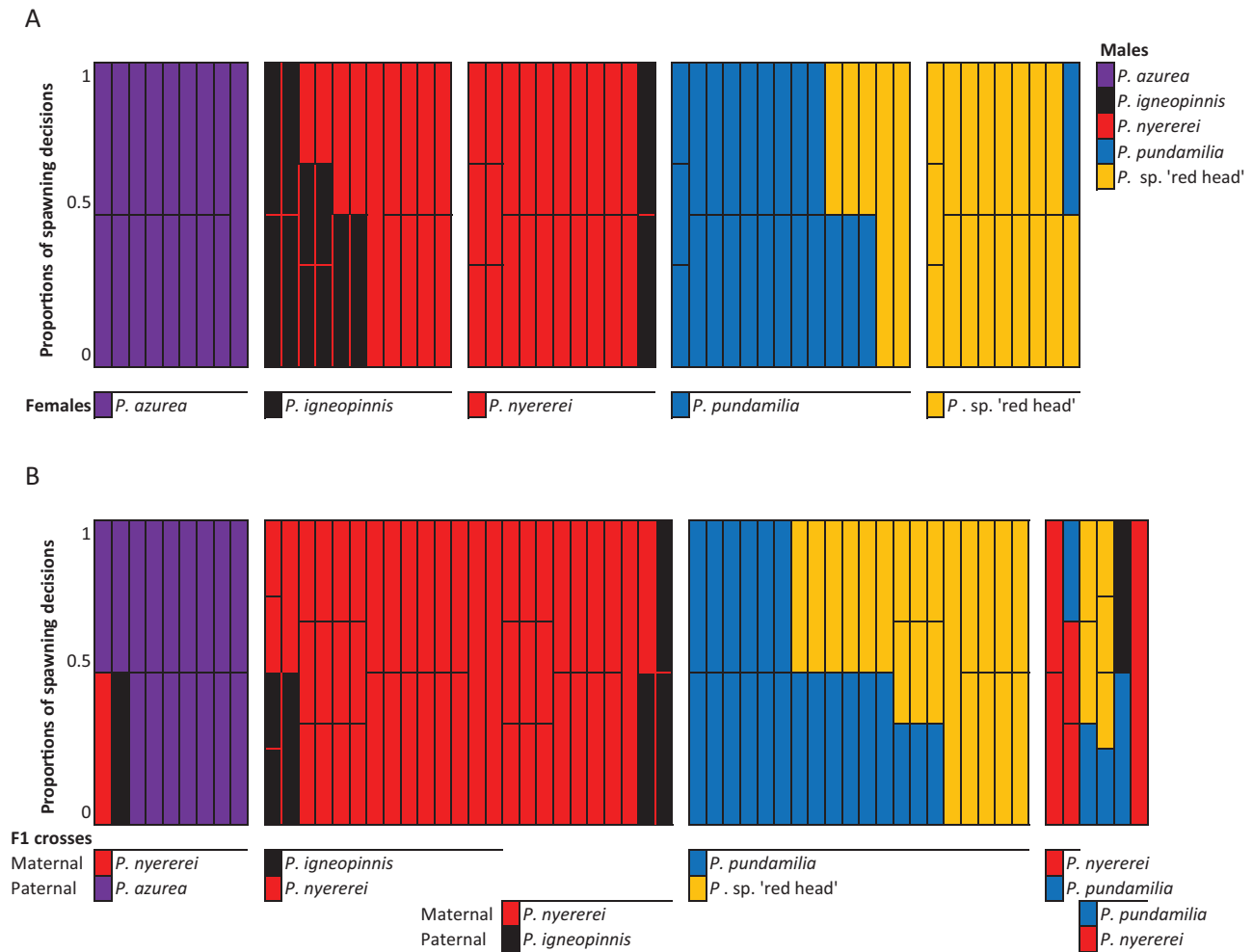
Figure 2. Spawning patterns of the females of the five *Pundamilia* species (A) and the four F1 crosses (B). Each bar represents the spawnings by one female and the species and crosses are indicated under the bars. Each box within a bar represents one spawning decision and its colour show the species the male who fathered the offspring belonged to. Paternities were assigned with microsatellite DNA analyses (Supplementary Tables S1–S3). Figure 1 and Supplementary Material S1 show male nuptial colouration and provide further information about the species.

the dorsal fin provided a DNA sample. Females were introduced when large enough to be PIT-tagged (size differences depended on age). For the first 3 months, only wild-type fish were present; F1 hybrid females and more wild-type fish were added over the following year. Females were stripped of embryos/juveniles once a month and released back into the experimental tank for a second and third clutch and thereafter removed. Females with eggs were placed in a separate aquarium until the eggs hatched. Larvae/juveniles were euthanized using MS-222 and stored in 95% ethanol prior to paternity analyses. Supplementary Table S1 shows when each female spawned, i.e., was present in the aquarium. The size of males was similar among species and initially 99–109 mm TL although they grew during the experiment. Female sizes when spawning were 65–97 mm TL and we confirmed for each female that it could pass the grids of the dividers.

Paternity analyses

Where possible, four embryos from each of two (sometimes three) clutches per female were genotyped at two to four microsatellite loci, Ppun5, Ppun7, Pun17, and Ppun21 (Taylor et al. 2003) using the same methods for DNA extraction and PCR reactions as in Svensson et al. (2017). The amplified

DNA samples were genotyped on a Beckman Coulter CEQ 8000 capillary sequencer. Genotypes were received from the CEQ 8000 Series Genetic Analyses System 8.0.52. Paternities were determined manually by inspection of the allele size estimates, and males that possessed two alleles at a microsatellite locus that were both not present in the offspring were excluded as possible fathers. In all analyzed offspring, paternity could be assigned with 100% certainty to one species only (Supplementary Tables S1–S3). If a clutch was confirmed to be fathered by more than one male, each sire was considered to represent one mate choice decision by the female.

Statistics

We tested for assortative mating among species pairs (ignoring any matings with third species) with pairwise Wilcoxon signed-rank tests (conspecific vs. heterospecific) of the number of spawning decisions. For pairs that did not show complete assortative mating, we used binary logistic generalized linear models (GzLMs) and Wald statistics to test whether females of such species differed in the degree of preference they showed for mating with conspecific males. GzLMs were set at "hybrid method" and scale parameter fixed at 1, with species as the factor, conspecific spawnings as the dependent variable, and the total number of

spawnings was the trial variable in which the event conspecific spawning could occur. The model reports the estimated marginal mean proportion and standard error (EMM ± SE).

We tested if hybrid females differed in preferences from females of the two parental species. For example, if females of the maternal species spawned with conspecifics only and females of the paternal species spawned with conspecifics and one other species, three species (6/10 males in the experiment) would be "preferred" by hybrids spawning as a combination of their parental species female preferences. By using the expected value of 0.6 in the binomial test, we test if the spawning patterns differ significantly from 6/10 spawnings with "preferred" males. The expected value was 0.4, 0.6, or 0.8 depending on the cross.

We tested for a bias in the preferences (i.e., maternal or paternal effects or dominance) using Wilcoxon signed-rank tests. In the *P. azurea* × *P. nyererei* cross, the ratio of available preferred males was 2:4. However, the bias in the data was towards spawning with *P. azurea* and we consider the test conservative. In the two cross-types in both directions (*P. igneopinnis* × *P. nyererei* and *P. nyererei* × *P. pundamilia*), we tested for differences using GzLMs as described above. Maternal species was a factor in the models.

We assume that our measures of individual female preferences are independent based on using two males of each species, changing the set of males, and in most cases, analyzing two clutches, although we have not controlled for male identity in the statistical analyses. All tests were two-tailed. Wilcoxon signed-rank tests and GzLMs were calculated in SPSS 27.0 whereas binomial tests were calculated in GrapPad Prism v. 9.3.1. using "method of small *p*-values" for exact double-sided testing.

Results

Paternity was determined for 838 offspring from 217 clutches of 114 females (Figure 2A and B, Table 1, Supplementary Tables S1 and S3).

Wild-type females

The spawning patterns of the wild-type females are summarized in Figure 2A and Table 1.

Females of *Pundamilia azurea*, which can occur sympatrically with all of the other four species, never mated with heterospecifics (Wilcoxon signed-ranks, all four pairwise comparisons $N = 9$, $Z = 2.887$, $p = .004$).

Pundamilia igneopinnis females showed no significant preference for conspecific males over males of the parapatric ecologically complementary *P. nyererei* (Wilcoxon signed-ranks, $N = 12$, $Z = 0.368$, $p = .71$), but never mated with any of the other three species (spawnings with *P. nyererei* omitted, Wilcoxon signed-ranks, all three comparisons $N = 8$, $Z = 2.598$, $p = .009$). *Pundamilia nyererei* females occasionally mated with male *P. igneopinnis*, but showed a significant preference for conspecifics (Wilcoxon signed-ranks, $N = 11$, $Z = 2.653$, $p = .008$). They never mated with any of the three other species (spawnings with *P. igneopinnis* omitted, Wilcoxon signed-ranks, all three comparisons, $N = 10$, $Z = 2.972$, $p = .003$). *Pundamilia nyererei* females were significantly more likely to spawn with conspecifics than *P. igneopinnis* females were (GzLM, proportional spawnings with conspecifics, *P. nyererei*: $N = 11$, 0.91 ± 0.06; *P. igneopinnis*: $N = 12$, 0.54 ± 0.10, $\chi^2 = 6.669$, $df = 1$, $p = .010$).

In the parapatric ecologically complementary species pair *P. pundamilia*—*Pundamilia* sp. "red head," females of both species mated a few times with males of the other species. Females of both species, nonetheless, mated significantly species assortatively (Wilcoxon signed-ranks: *P. pundamilia*: $N = 14$, $Z = 2.787$, $p = .005$; *Pundamilia* sp. "red head": $N = 9$, $Z = 2.714$, $p = .007$). Again, females never mated with any of the other three species (*P. pundamilia* females, with spawnings with *Pundamilia* sp. "red head" omitted, Wilcoxon signed-ranks, all three comparisons $N = 13$, $Z = 3.275$, $p = .001$; *Pundamilia* sp. "red head" females, spawnings with *P. pundamilia* omitted, Wilcoxon signed-ranks, all three comparisons $N = 9$, $Z = 2.807$, $p = .005$). There was no significant difference between the females of these two species in their tendency to mate with conspecifics over the other species within the pair (GzLM, proportional spawnings with conspecifics, *P. pundamilia*: $N = 14$, 0.79 ± 0.08, *Pundamilia* sp. "red head," $N = 9$, 0.94 ± 0.05, $\chi^2 = 1.784$, $df = 1$, $p = .19$).

F1 hybrids

The spawning patterns of the F1 hybrid females are summarized in Figure 2B. F1 hybrid females mated with males of all species that were accepted as mates by females of their two parental species. Strikingly, no F1 hybrid female mated with a male of a species that was not also accepted by the females of its parental species (Figure 2B). This was significant for three crosses (binomial tests, *P. nyererei* × *P. azurea*, test proportion 0.6, $N = 9$, $p = .014$; *P. igneopinnis* × *P. nyererei*, test proportion 0.4, $N = 24$, $p < .001$; and *P. pundamilia* × *Pundamilia* sp. "red head," test proportion 0.4, $N = 20$, $p < .001$). In the *P. nyererei* × *P. pundamilia* cross, the sample size was too low ($N = 6$) and the test proportion was too high (0.8) to reject random mating (binomial test, $p = .61$). The production of backcross fry in all crosses showed that the females of the F1 crosses were fertile.

In the *P. nyererei* × *P. azurea* cross, there was a significant bias towards mating with the paternal species, *P. azurea* (Wilcoxon signed ranks, $N = 9$, $Z = 2.646$, $p = .008$). In the *P. pundamilia* × *Pundamilia* sp. "red head" cross there was no bias in preferences (Wilcoxon signed-ranks, $N = 20$, $Z = 0.406$, $p = .68$). The other two crosses were in both directions. In the *P. igneopinnis* × *P. nyererei* cross, spawning preferences were unaffected by maternal species (GzLM, proportional spawnings with *P. nyererei*, *P. igneopinnis* mother: $N = 14$, 0.91 ± 0.05, *P. nyererei* mother: $N = 10$, 0.86 ± 0.07, $\chi^2 = 0.277$, $df = 1$, $p = .59$). There was a strong preference for spawning with *P. nyererei* (Wilcoxon signed-ranks, $N = 20$, $Z = 3.78$, $p < .001$), hence, there were no signs of maternal imprinting in these three crosses. In the *P. nyererei* × *P. pundamilia* cross, hybrid females chose more similar spawning decisions to their maternal species than to their paternal species (GzLM on proportional spawnings with *P. nyererei* and *P. igneopinnis*, *P. nyererei* mother: $N = 2$, 0.80 ± 0.18, *P. pundamilia* mother: $N = 4$, 0.20 ± 0.12, $p = .043$).

The colours of the males in the F1 hybrid crosses are shown in Supplementary Figure S2. Approximately a quarter of the males of both red dorsum vs. melanic crosses lacked red nuptial body colouration (Figure 2B, Supplementary Figure S2).

Discussion

In the present study with species representing each major male nuptial colour type of Lake Victoria cichlids (Seehausen

et al., 1999), all species with sympatric distributions mated fully assortatively in the laboratory setting, which suggests that assortative mate choice can be maintained independently of microhabitat and other environmental factors. The two species pairs that showed partial assortative mating in our experiments are parapatrically distributed in the lake and ecologically similar (depth and diet preferences) with species belonging to the same (red dorsum, one melanized) or different (blue vs. red chest) male nuptial colour pattern. Hence, mating and ecology both suggest that these complementary species may not be able to co-exist sympatrically in the absence of other ecological mechanisms (Irwin & Schluter, 2022; Mittelbach & McGill, 2019). However, other species pairs that are not known to occur in sympatry showed complete assortative mating. Our experiments with interspecific hybrid females corroborated the results from the nonhybrid females by these females mating with males of all those species that were accepted as mates by females of their parent species but not with any others. Although several non-exclusive scenarios might explain these results, it is important to point out that present-day mate preferences, regardless of their evolutionary origin, may affect species (co)distributions.

Where speciation is mainly parapatric or allopatric, female preferences may determine the outcome of secondary contact or range expansion: only species with divergent female preferences and male traits can co-exist in sympatry (Irwin & Schluter, 2022; Rhymer & Simberloff, 1996; Seehausen & Schluter, 2004). Female mating preferences may thus help explain empirical field observations that show that geographically parapatric species do occasionally appear at each other's islands, but fail to establish populations in the presence of the other species (Seehausen, 1996). Three of our test species (*P. azurea*, *P. nyererei*, and *P. pundamilia*) do occur in sympatry at several islands. No mating occurred between these in our experiments. Furthermore, while we had sampled *P. nyererei* and *P. pundamilia* from Makobe Island (where *P. azurea* is absent), our *P. azurea* population came from Ruti Island, that these populations per se may not be reinforced regarding each other. The other two species are parapatric with at least one of the previous species: the distributions of *P. nyererei* and *P. igneopinnis* are mutually exclusive and ecologically similar, as are the distributions of *P. pundamilia* and *Pundamilia* sp. "red head" (Seehausen, 1996). Occasional individuals are found within the range of the complementary species (Feller et al., 2020; Seehausen, 1996). However, these complementary species appear to have overlapping heritable mate preferences preventing coexistence. For example, *P. nyererei* has been observed at Igombe Island, where *P. igneopinnis* is abundant, and the presence of intermediate-looking individuals subsequent to the arrival of *P. nyererei* was suggested to indicate hybridization (Seehausen, 1996). Subsequent surveys have failed to record *P. nyererei* at this site, suggesting the population was not established (Seehausen, unpublished data). This is consistent with the random mating of *P. igneopinnis* females between males of these species in our laboratory trials. In a previous multi-way mating trial, Seltz et al. (2016) reported on allopatric and parapatric *Pundamilia* populations of the same major nuptial colour type, "red dorsum." Mating patterns ranged from random between two conspecific populations, to strong but incomplete for females of *P. nyererei* choosing between *P. nyererei* and *P. igneopinnis* males (the reverse direction was not tested). Theoretical work by Irwin and Schluter (2022), suggested that ecologically divergent species whose hybrid offspring had low or no survival were unable to coexist unless a preference for conspecific mates was strong (tenfold in the model). Without low hybrid survival, preference for conspecific mates had to be extremely strong (hundredfold in the model). However, F1 offspring in our experiment had high survival until maturity and the females produced offspring in the experiment. Hybrids between Lake Victoria cichlid species are generally known to have high viability and fertility (Stelkens et al., 2010, 2015). In the context of Irwin and Schluter's model, this implies a need for coexistence to have very strong assortative mating, unless there is lower relative hybrid fitness through extrinsic post-zygotic barriers in their natural environment. Thus, the complex and often mosaic distribution patterns of cichlid fish species in Lake Victoria may not be just due to barriers to dispersal across unfavourable habitats, but may often result from variation and divergence in female mate preference and male nuptial traits. In addition, male-male competition has been suggested to stabilize the coexistence of *Pundamilia* species (Dijkstra et al., 2010) and, although overlapping, there are differences in breeding sites (Seehausen, 1996; Seehausen et al., 1998a), parasite communities (Maan et al., 2008), microhabitats, and diets (Bouton et al., 1997; Maan et al., 2008; Seehausen, 1996; Seehausen et al., 1998a) which potentially act in concert with dispersal barriers and female preferences in promoting or preventing coexistence in *Pundamilia* species.

Because many alleles involved in female mate preferences and male nuptial traits likely stem from standing genetic variation being transmitted between species through introgressive hybridization (Meier et al., 2017a), similar traits and preferences in different species may even be caused by similar combinations of alleles. A simple genetic basis for mating preferences and corresponding male nuptial colour could facilitate stable phenotypic differentiation and make it more robust during gene flow (Gavrilets & Vose, 2005; Yeaman & Otto, 2011; Yeaman & Whitlock, 2011). In *Pundamilia* sp. "nyererei-like" and *Pundamilia* sp. "pundamilia-like," the strong female preference for conspecifics appears to be oligogenic with few genes that have major effects (Haesler & Seehausen, 2005; Svensson et al., 2017). Furthermore, their male nuptial colour patterns "red dorsum" vs. "blue" is likely oligogenic (Feller et al., 2020; Magalhães & Seehausen, 2010), with significant quantitative trait loci (QTLs) that have moderate to large effects on red and yellow (Feller et al., 2020). These young species differ in many genomic regions that also distinguish the ancestral *P. pundamilia—P. nyererei* pair investigated in the present study (Meier et al., 2018). These may include genes influencing assortative mating. In contrast, the study of the cross between *P undamilia* sp. "red head" and *P. pundamilia* from the present study failed to detect any QTLs for the red/yellow chest colouring, perhaps suggesting a more polygenic basis to the nuptial colour differences between these complementary species without large effect genes (Feller et al., 2020). Thus, hypothetically, patterns of coexistence may be influenced by which sexual signalling and mate preference traits allopatric lineages "end up" with.

An alternative scenario is the reinforcement of premating isolation in secondary contact following range expansion or shift. For mating preferences to become reinforced in the face of gene flow, pre-existing mating preferences must already be relatively strong when secondary contact is first established, and the fitness of hybrids has to be relatively low too (Coyne & Orr, 1989; Irwin, 2020; Liou & Price, 1994). However,

Van der Sluijs et al. (2008) found no differences in fecundity, fertility, sex ratio, or growth rates between *Pundamilia* species and their hybrids. There is also little evidence for extrinsic ecological selection against hybrids between young sympatric *Pundamilia* species, although selection against intermediate morphologies was shown in the oldest pair, *P. pundamilia* and *P. nyererei* at Makobe Island (van Rijssel et al., 2018). This suggests that assortative mating can evolve without ecological or intrinsic selection against hybrids, possibly as a by-product of adaptation to light regimes (Seehausen et al., 2008).

The direction of asymmetric assortative mating can be indicative of the action of reinforcement. The beginnings of reinforcement should be seen most readily in the species in a hybridizing pair that receives more migrants from the other species (Yukilevich, 2012). In our case, *P. igneopinnis* at Igombe repeatedly experiences an influx of and introgression from *P. nyererei* from Makobe Island, whereas *P. nyererei* at Makobe does not seem to experience contact with *P. igneopinnis*. If reinforcement were operating, we would expect *P. igneopinnis* females to mate more strongly assortatively than *P. nyererei* females, but we observed the opposite which may be explained by the introgression of *P. nyererei* preference genes or by ancestral retention of shared mating preferences where the differences in male nuptial colouration could be due to adaptation of the signals to match the same preference in different signalling environments. If reinforcement were operating, we would expect the strength of assortative mating to be elevated in populations from the same island within a sympatric range. However, assortative mating was independent of whether the populations we tested actually had a sympatric origin or not: Females of *P. azurea* from Ruti Island mated exclusively with conspecifics even though the populations of sympatric species in our tests (*P. nyererei*, *P. pundamilia*, *Pundamilia* sp. "red head") originated from other islands (Makobe and Zue). Neither of these localities is inhabited by *P. azurea*. Reinforcement could therefore not have operated locally within these populations. Finally, F1 hybrid females, whether in crosses between complementary or sympatric species, mated freely with males of all species accepted by females of either of their parental species and we found no support for any inherited avoidance.

Sympatric species generally show strong assortative mating whereas results from parapatric and allopatric species are mixed. For example, Hendry et al. (2009) emphasized that in *Gasterosteus* sticklebacks, strong behavioural assortative mating has only been demonstrated among sympatric species pairs, although introgression happened there too. This assortative mating is primarily due to by-product mechanisms (Rundle et al., 2000). Other systems are more complex. In *Heliconius* butterflies, assortative mating and other premating barriers are strong among sympatric species, although introgression can occur: visual signals are also important in mate choice within and among species, and there is the additional complication of selection for signal similarity in mimicry rings formed under predation pressure (Merot et al., 2017). Complete assortative mating has been observed in trials of sympatric species of Lakes Malawi and Tanganyika haplochromines *Maylandia*, *Petrochromis*, and *Tropheus* (Ding et al., 2014; Knight & Turner, 2004; Plenderleith et al., 2005; Wagner et al., 2012) and there are observations of complete assortative mating between recently diverged allopatric species of *Labeotropheus*, *Lethrinops*, and *Rhamphochromis* spp. (Genner et al., 2007; Pauers et al., 2010; Tyers et al.,

2014). However, conspecific populations or complementary species from different islands generally mate partially assortatively, or randomly (Knight & Turner, 1999; Nyalungu & Couldridge, 2020; Zoppoth et al., 2013). Likewise, allopatric populations of swamp-dwelling *Astatotilapia calliptera* showed significant, but incomplete assortative mating when male colours were divergent, but random mating when colours were similar (Tyers & Turner, 2013).

A lack of strong assortative mating among blue and red varieties of *Tropheus moorii* seems to have resulted in the formation of a yellow hybrid population at an intermediate location during the Pleistocene megadroughts (Sefc et al., 2017). A more recent example is previously parapatric *Phaulacridium* grasshoppers where recent expansions in relation to agricultural intensification have resulted in the formation of hybrid swarms in sympatry (Morgan-Richards et al., 2022). The measured incomplete assortative mating (Morgan-Richards et al., 2022) may explain the parapatric distributions in this system. Other examples come from accidental or purposeful translocations of species into the range of related species. If they have overlapping preference functions, hybridization may lead to the merging of gene pools and loss of species distinctiveness (Blackwell et al., 2020; Egger et al., 2008; Genner et al., 2006) as was the case when a hybrid swarm replaced the pupfish *Cyprinodon pecosensis* in Pecos River in Texas after a few *Cyprinodon variegatus* were introduced (Echelle & Connor, 1989; Rosenfield & Kodric-Brown, 2003).

Hence, as expected (Irwin & Schluter, 2022; Rhymer & Simberloff, 1996; Seehausen & Schluter, 2004), sympatric species must show high degrees of assortative mating to be able to coexist as distinct species. Indeed, Weir and Price (2011) inferred range expansion into secondary sympatry in 418 species of New World Birds to be slowed down by a low rate of accumulation of reproductive isolation. However, recently diverged allopatric species may also show high degrees of assortative mating, which potentially allow coexistence should these species become sympatric.

Conclusions

The present study expands our knowledge of the mate preferences among Lake Victoria's rocky shore cichlids of the genus *Pundamilia*. We find that females of species that occur sympatrically in nature consistently show strong conspecific mate preferences while preferences in parapatric and allopatric pairs range from none to strong. We suggest that mate choice functions may have an important influence on whether species have sympatric distributions among Lake Victoria cichlid fish. Together with recent theoretical advances, our result supports the notion that mating preferences of potentially crossbreeding species need to be included in coexistence theory.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The data are available in Figure 2A and B. The paternity analyses are available in electronic supporting information (Supplementary Tables S1–S3). The data underlying this

article are also available in DRYAD (https://doi.org/10.5061/dryad.fqz612jxx, Svensson et al., 2023).

Conflicts of interest

We declare that we have no conflict of interest.

Ethical statement

This work was ethically reviewed and performed under a UK Government Home Office Licence (PPL 60/3295). O.Se. thank the Tanzanian Commission for Science and Technology for a research permit and the Tanzanian Fisheries Division for a permit to export live fish.

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