Phenotypic divergence but not genetic distance predicts assortative mating among species of a cichlid fish radiation

R. B. STELKENS*†‡ & O. SEEHAUSEN†‡

*Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland
†Division of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, Bern, Switzerland
‡Department of Fish Ecology and Evolution, Centre of Ecology, Evolution and Biogeochemistry (CEEB), Eawag Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland

Abstract

The hypothesis of ecological divergence giving rise to premating isolation in the face of gene flow is controversial. However, this may be an important mechanism to explain the rapid multiplication of species during adaptive radiation following the colonization of a new environment when geographical barriers to gene flow are largely absent but underutilized niche space is abundant. Using cichlid fish, we tested the prediction of ecological speciation that the strength of premating isolation among species is predicted by phenotypic rather than genetic distance. We conducted mate choice experiments between three closely related, sympatric species of a recent radiation in Lake Mweru (Zambia/DRC) that differ in habitat use and phenotype, and a distantly related population from Lake Bangweulu that resembles one of the species in Lake Mweru. We found significant assortative mating among all closely related, sympatric species that differed phenotypically, but none between the distantly related allopatric populations of more similar phenotype. Phenotypic distance between species was a good predictor of the strength of premating isolation, suggesting that assortative mating can evolve rapidly in association with ecological divergence during adaptive radiation. Our data also reveals that distantly related allopatric populations that have not diverged phenotypically, may hybridize when coming into secondary contact, e.g. upon river capture because of diversion of drainage systems.

Introduction

The efficacy of ecological selection to cause divergence in the face of gene flow is one of the fundamental debates in speciation research. Speciation is thought to most often result from population divergence in the presence of physical impediments to gene flow (Mayr, 1963; Turelli *et al.*, 2001) where post-zygotic isolation builds up as a byproduct of the genetic differences accumulating in diverging populations because of drift (Gavrilets & Boake, 1998) and selection (Dobzhansky, 1951; Coyne & Orr, 2004). Classical theory of reinforcement (Dobzhansky, 1951; Noor, 1995, 1999; Servedio, 2001, 2004) predicts that upon secondary contact, premating isolation can then become reinforced in response to selection against hybrids suffering from low fitness due to genetic incompatibilities that have accumulated in the allopatric phase. Theoretical (Gavrilets, 2003) and experimental work (Rundle *et al.*, 1998; Mooers *et al.*, 1999; Rundle, 2003) cast doubt on drift being a common cause of speciation, and much interest in the role of divergent natural and sexual selection and 'ecological speciation' has emerged (Schluter, 1996, 2000, 2001; Rundle & Nosil, 2005). Herein, premating incompatibility can arise as a byproduct of divergent ecological selection on traits that affect mate choice via pleiotropy (Kilias *et al.*, 1980; Dodd, 1989; Vines & Schluter, 2006), through divergent selec-

Correspondence: Rike Stelkens, Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland.
Tel.: +41 21 692 4249; fax: +41 21 692 4165;
e-mail: rike.stelkens@unil.ch

tion directly acting on (female) mating preferences, e.g. when the conspicuousness of a (male) sexual trait varies with the signalling environment (Endler, 1992; Schluter & Price, 1993; Boughman, 2002; Maan *et al.*, 2006; Seehausen *et al.*, 2008), or through direct selection for assortative mating. In the latter case, reduced exogenous fitness of intermediate genotypes exhibiting a poor fit to both parental environments, can lead to the evolution of assortative mating through ecologically based, reinforcement-like mechanisms, the effectiveness of which has been explored in several models (Kondrashov & Kondrashov, 1999; Doebeli & Dieckmann, 2003; Gavrilets, 2004; Kawata *et al.*, 2007; Leimar *et al.*, 2008). The number of cases where reproductive incompatibilities between populations are thought to have accumulated as a result of ecological divergence has increased considerably in recent years, e.g. in fish (Rundle *et al.*, 2000; Boughman, 2001; Schliewen *et al.*, 2001; McKinnon *et al.*, 2004; Terai *et al.*, 2006; Seehausen *et al.*, 2008), insects (Funk, 1998; Caillaud & Via, 2000; Jiggins *et al.*, 2001; Nosil & Crespi, 2006; Nosil, 2007), other animals (Podos, 2001; Funk *et al.*, 2006) and plants (Ramsey *et al.*, 2003; Friar *et al.*, 2006; Baldwin, 2007; Martin & Willis, 2007). In systems where phenotypic and genetic divergence vary independently, e.g. in adaptive radiations where changes in ecomorphology are not necessarily correlated with the genetic distance between species, the ecological speciation hypothesis predicts that phenotypic distance is the stronger predictor of assortative mating (McPeek & Wellborn, 1998). In this paper, we test this hypothesis in cichlid fish of a young and previously unknown adaptive radiation.

As long as divergent selection outweighs gene flow, ecological adaptation can cause rapid changes in the mating system (Schluter, 2001). One might hence predict that selection-driven evolution of mating preferences is prominent and dominates over other mechanisms during adaptive radiations, when multiple available niches are colonized in short succession by the same ancestral population. The ideal study system to test this prediction is one that comprises populations having evolved in geographical isolation but under similar ecological selection regimes, and also populations having evolved in an environment without geographical barriers but with different selection pressures between alternative niches. We recently discovered such a system in the upper Congo (Zambia/DRC). The cichlid fish *Pseudocrenilabrus philander* is geographically widely distributed in rivers, swamps and lakes of South and East Africa. Most populations are ecologically unspecialized, and phenotypically similar. Such populations occupy the upper Luapula/Congo River system and Lake Bangweulu. Yet, in nearby Lake Mweru (Zambia/DRC) a population coexists with a diverse and phylogenetically young adaptive radiation that emerged from the same species.

Pseudocrenilabrus takes a basal position in the phylogeny of haplochromine cichlids, a group that comprises all large African cichlid fish radiations except some of those in Lake Tanganyika (Joyce *et al.*, 2005; Katongo *et al.*, 2005; Koblmueller *et al.*, 2008). *Pseudocrenilabrus* has been described as a species-poor genus that, in contrast to some other haplochromines, did not radiate into sympatric species (Katongo *et al.*, 2005). It currently comprises three nominal species (*P. philander*, *P. nicholsi* and *P. multicolor*). Of these, *P. philander* has the widest geographic distribution throughout South-Central, South-East and South Africa. It contains several subspecies and shows strong biogeographic sub-structuring (Skelton, 1991). This species and both others are morphologically rather unspecialized, ecologically generalized cichlids of small body size (adult males are 5–8 cm, females are 4–6 cm long) that typically inhabit slow flowing parts of rivers, swamps and sink holes (Greenwood, 1989). They are generally thought of as poor dispersers. In lakes, they are typically restricted to the marginal habitats, i.e. shallow shores with sandy or muddy bottoms and dense vegetation where they feed on insect larvae, detritus and algal material. Apart from Koblmueller *et al.* (2008), who described a population from a small tributary of Lake Tanganyika containing two closely related but genetically distinct colour morphs, which they suggest are currently undergoing incipient speciation, there was no evidence for sympatric or parapatric species diversity anywhere within the genus.

During fieldwork on Lake Mweru, we discovered at least 13 distinct phenotypes of *Pseudocrenilabrus* within the lake, differing in size, body shape, jaw morphology and male colouration, most exhibiting novel phenotypes not reported from anywhere else in the large distribution range of the genus. Strikingly, most of them occur in sympatry. Comparative analysis of morphometric distances reflecting variation in ecologically relevant shape elements, revealed that this *Pseudocrenilabrus* species flock diversified in phenotypic dimensions resembling those of the classical cichlid adaptive radiations (R.B. Stelkens *et al.,* unpublished data). Yet, phylogenetic reconstruction revealed that there are only two major mitochondrial haplotype lineages in *P. philander*, one of which is endemic to Lake Mweru while the other one is widely distributed in lakes and rivers of East and Southern Africa, including Lake Mweru and Lake Bangweulu in the same drainage system (Stelkens *et al.,* in preperation). Lake Mweru was apparently colonized by both of these deeply divergent lineages, but while one diversified rapidly and strongly dominates in all the endemic species, the other lineage went almost extinct in the lake.

The different geographic populations of *P. philander*, together with the adaptive radiation in Lake Mweru, provide the rare opportunity to compare effects of strong genetic but weak ecological and phenotypic divergence in geographical isolation with effects of strong ecological and phenotypic despite weak genetic divergence on reproductive compatibility.
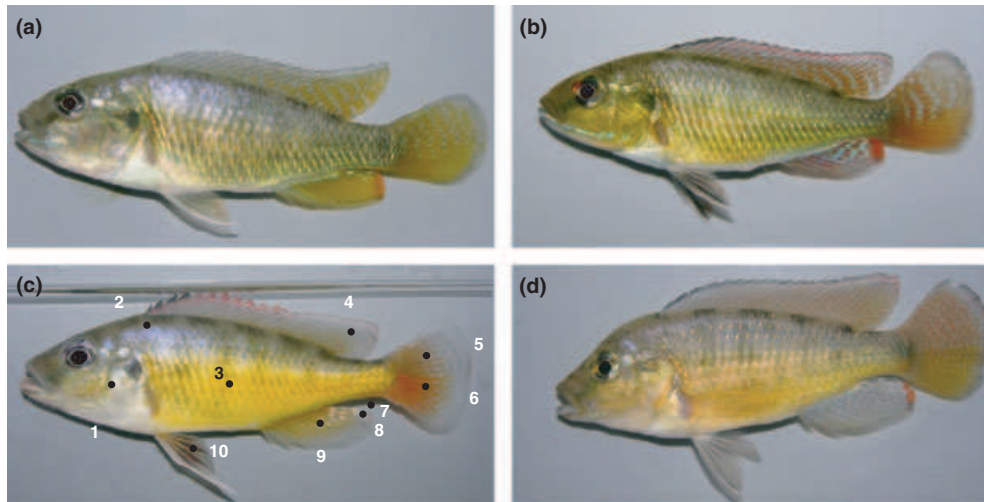
Fig. 1 Males of the four groups tested in mate choice experiments: (a) *Pseudocrenilabrus philander* from Lake Bangweulu, a distantly related generalist that colonized Lake Mweru; (b) *P. philander* from Lake Mweru, a generalist representing the second lineage colonizing Lake Mweru; (c) *P.* sp. *grey back*, a closely related, but ecologically derived type from Lake Mweru; d) *Pseudocrenilabrus* sp. *weed picker*, another closely related, but ecologically derived type from Lake Mweru. Figures (b–d) are sympatric, while (a) is allopatric to all. On (c), the position of all 10 landmarks used for visual scoring of male colouration is indicated.

We conducted mate choice experiments in the laboratory using four different groups: (i) an allopatric *P. philander* population from Lake Bangweulu, representing one of the two mitochondrial lineages that seeded the Lake Mweru radiation and presumably resembles the ancestral phenotype (Fig. 1a); (ii) a sympatric *P. philander* population from Lake Mweru, representing the other mitochondrial lineage that seeded the radiation and also resembles the ancestral phenotype (Fig. 1b); (iii) a sympatric species (*Pseudocrenilabrus* sp. '*grey back*') from Lake Mweru, representing an ecologically derived phenotype (Fig. 1c); (iv) another sympatric species (*Pseudocrenilabrus* sp. '*weed picker*') from Lake Mweru (Fig. 1d), representing another ecologically derived phenotype with a different ecological specialization than (c). The three members of the adaptive radiation (ii, iii and iv) are genetically closely related to each other while the allopatric *P. philander* population (i) represents a genetically distant lineage. The radiation members differ distinctly in male breeding colouration, whereas the Lake Bangweulu *P. philander* resembles the Lake Mweru *P. philander*.

First, we tested for assortative mating among the three phenotypically and ecologically divergent putative species from Lake Mweru (ii, iii and iv). Then we tested the two allopatric *P. philander* against each other (i vs. ii). If loss of premating compatibility resulted from evolution in geographical isolation, we would predict stronger assortative mating between the genetically divergent populations from different lakes. On the other hand, if loss of premating compatibility resulted from divergent adaptation, we would predict elevated levels of assortative mating among the sympatric incipient species.

Materials and methods

Experimental animals

The four populations were collected in Lakes Mweru and Bangweulu in September 2005. Sampling occurred on several days using beach seine nets covering a large area (approximately 100 m of lake shore). Both lakes are part of the Upper Congo River system and connected by the Luapula River that drains Lake Bangweulu in the south, then bends northwards and feeds into Lake Mweru through large swampy areas (Fig. 2) (Jackson, 1961). Although the linear distance between the lakes is only 250 km, the length of the connecting river is approximately 550 km.

From each lake, we collected one population of *P. philander* (Skelton, 1991). The two populations are phenotypically similar to each other and match the typical appearance of *P. philander* across much of South and East Africa (Fig. 1a,b). Both have bright yellow–green flanks and ventra with a greyish to brownish dorsum. The anal fins of territorial males are intensely blue with rows of transparent-white or orange–red speckles on the membranes. Dorsal and caudal fins are, to varying degrees, yellow, red and orange and also show speckles. The pelvic fins turn black when males are territorial. Males are further characterized by a bright sky blue line extending from the lower lip across the cheeks to the operculum, a trait widely distributed across the geographical range of the species. Females share the overall colour of the males but are less intense making them appear pale brownish or grey. The only

Fig. 2 Map of the sampling region Lakes Mweru and Bangweulu. Red dots indicate the two collection sites. The riverine connection between the lakes, the Luapula River, was coloured light blue for better visibility. The run of the river experiences seasonal changes.

conspicuous difference between the two populations is that males from Lake Mweru display a bright red spot on the trailing edge of the anal fin, while this spot is bright orange in males from Lake Bangweulu.

Two new types of *Pseudocrenilabrus*

Pseudocrenilabrus sp. '*grey back*' and *Pseudocrenilabrus* sp. '*weed picker*' occur in sympatry with *P. philander* in Lake Mweru, but they differ strikingly in morphology and colouration (Fig. 1c,d). *Pseudocrenilabrus* sp. '*weed picker*' was caught in a reed belt in the littoral zone. *Weed picker* has a different head morphology than any of the other types studied here. While *philander* has a curved head profile, *weed picker* has a pointy snout. In comparison to cichlids in the larger African radiations, this indicates that it may pick insect larvae from between and on the vegetation. *Weed picker* males display bright orange–yellow flanks and only a thin red spot on the trailing edge of the anal fin.

Pseudocrenilabrus sp. '*grey back*' was collected further offshore in deeper waters with no emergent vegetation. It

is easily and consistently identified by its streamlined body shape, a thinner, elongated caudal peduncle, a conspicuously shaped 'horse-head' with a deep dorsal head profile, and strikingly different breeding colouration. Nuptial males have bright yellow flanks without the greenish hue of *philander* and a characteristically metallic-grey dorsum, sharply defined against the yellow flanks. The lower anterior half of the caudal fin is conspicuously orange. *Grey backs* lack the characteristic blue anal fin and blue lower lip of *philander*. Their anal fin is mainly transparent with a yellow hue and only a very thin line of red on the trailing edge. The body shape together with the habitat where *grey backs* were caught, suggest that it is an offshore demersal feeder. Females of both 'new' types resemble the males morphologically. In the case of *grey back*, females show the same distinctly metallic-grey dorsal colouration but it extends over all of the body.

To query the different types for their mating preferences in the laboratory, live fish of all four groups (*philander* Bangweulu, *philander* Mweru, *grey back* Mweru and *weed picker* Mweru) were shipped to our laboratory at EAWAG, Switzerland.

Experimental design

Experiments were carried out in $196 \times 40 \times 40$ cm large aquaria, divided into three equally sized compartments (Fig. 3). To measure female mating preferences based on actual spawning events, we used the 'egg catcher' experimental design described in Kidd *et al.* (2006) and previously applied to assess cichlid mate choice (Nelson, 1995; Plenderleith *et al.*, 2005). The design allows for full completion of spawning in two-way choice experiments through clear, ultraviolet-transparent Plexiglas walls separating the female compartment in the middle from the two adjacent male compartments to the left and right. The walls were perforated with holes of 0.5 cm diameter every 2 cm², allowing females to assess the full sensory repertoire of male mating cues, including chemical (Plenderleith *et al.*, 2005) and visual cues (Seehausen & Van Alphen, 1998) as well as sound (Amorim *et al.*, 2004). Directly in front of the two walls, inside of the female compartment, egg collectors were installed as rectangular boxes running across the entire depth of the tank. The collectors were covered with plastic mesh with holes large enough to allow eggs to fall through during spawning. These egg repositories allowed the observer to assign egg counts to the male behind the transparent wall. The male that obtained the larger number of eggs was considered the preferred sire. Male to male aggressive behaviour was physically constrained, thus not interfering with female mate choice decisions (Morris *et al.*, 1992; Kodric-Brown, 1993). We constructed spawning shelters in both male compartments using four bricks; two serving as walls and the other two as floor and roof leaving two sides open, one side for the male to
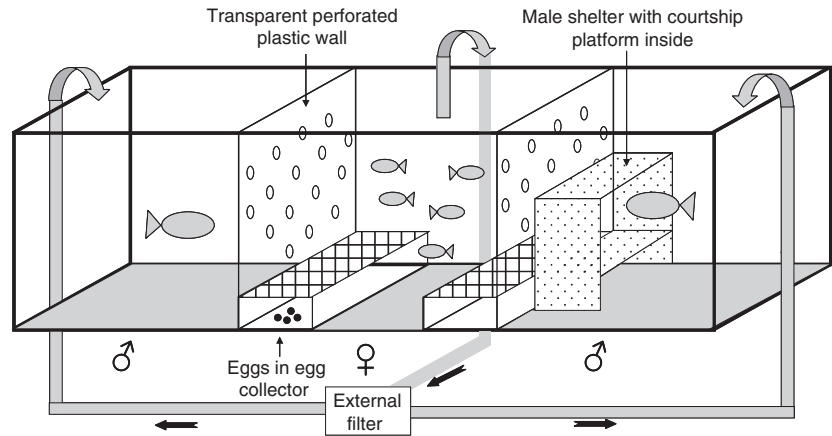
Fig. 3 Experimental design. Egg collectors were perforated with holes big enough to allow eggs to fall through during spawning. Only one spawning shelter is shown for illustrative purposes, but shelters were present in both male compartments. The direction of water flow is indicated by arrows.

enter the cave and the other side facing the female compartment and the egg collector. The bottom of the male shelter was at level with the plastic mesh covering the egg collectors on the other side of the perspex wall, forming a platform for the courtship display preceding each spawning event.

Water circulation in the tank was manipulated such that water constantly passed through the perforated plastic walls from both male compartments into the middle compartment, from where it was pumped out and into an external Eheim aquarium filter, and back into the tank through two separate inflows, one in each male compartment (Fig. 3). In this way, females were exposed to water enriched with pheromones of both males. Once a week, one-third of the water was exchanged by tap water. Water temperature was kept constant at 24–25 °C and fish were kept on a 12L : 12D light regime using standard aquarium fluorescent light tubes with full spectrum lighting. Fish were fed daily with flakes and twice a week with a blend of fresh food made from shrimps, peas and *Spirulina* powder. The bottom of all three compartments was covered with a 2-cm thick layer of aquarium sand.

Experimental procedure

Four different experiments were conducted between May 2006 and January 2008. The first two experiments tested for assortative mating between the Lake Mweru population of ancestral phenotype and the two derived Lake Mweru species, all of them sympatric and members of the endemic radiation: Experiment 1, *philander* Mweru vs. *grey back*; Experiment 2, *philander* Mweru vs. *weed picker*. The third experiment tested for assortative mating between the two derived members of the endemic radiation: Experiment 3, *grey back* vs. *weed picker*. The fourth experiment tested for assortative mating between the allopatric populations of the ancestral phenotype; Experiment 4, *philander* Mweru vs. *philander* Bangweulu.

All males and females used in this study were first generation offspring (F1) of wild caught fish (every F1

family was derived from a different male–female combination), except for Experiment 2 where only wild caught fish were used. Prior to the experiments females had never seen the specific males they were offered, and experimental males and females never were siblings. Females had no prior experience with heterospecific males, but were raised in stock tanks with their brothers.

Females were introduced in the middle compartment 2 days before one male was put into each of the adjacent compartments. Only females of one of the two species tested were used per trial. To facilitate behavioural acclimatization with minimum levels of aggression, females were introduced to the experimental tank in groups of 3–14 individuals, all of which were from the same sib group. After a spawning had occurred, all females of that sib group were removed from the experiment and replaced by a different sib group of females. Sib groups were only used once and only spawnings of females from different families were considered genetically independent data points. It is in principle possible that an egg clutch derived from more than one female, i.e. if one spawning was closely followed by another, but it is unlikely that this would have gone unnoticed as we watched spawnings carefully and checked for eggs at least three times a day (morning, noon and evening). Also, female spawners were identified during or immediately after spawning, and in no case did more than one female show the symptoms of a recent spawning event. Female spawners were identified by inspecting the ventral region (convex before, concave after spawning) and the genital pore, which swells and protrudes before and during oviposition. Occasionally, a female was found with some eggs in her mouth. These eggs were not used for analysis as we were not able to determine with which male the female had spawned.

The position of male types in the aquarium was randomized over trials. Across all experiments, eggs were laid on average every 9.3 days. As soon as eggs were discovered in one or both egg collectors and after allowing sufficient time to ensure that the spawning

was completed, eggs were removed and both males were exchanged against two new males of the same type, that were not brothers of the preceding males. Only in one case (Experiment 1), not enough *grey back* males were available and one male was used in three different trials, but paired with a different opponent in each case. Upon removal, males were measured with a ruler to the nearest millimetre to control for size effects, before they were put back into separate stock tanks.

In Experiment 2, only wild caught males and females of unknown pedigree were used. Due to the sampling method we used to catch these fish (several days of beach seining covering a large sampling area), it is unlikely that they were from the same family. Hence, in Experiment 2, every female was allowed to spawn before it was removed from the experiment together with the two males, which were replaced by a new pair of males as described above. Here, all spawnings were treated as genetically independent data points.

Analysis of mate choice data

The data from the mate choice experiments were tested against the null hypothesis of random mate choice. We used paired *t*-tests on the number of eggs a female spawned with each male. To relax the normality assumption, we also applied Wilcoxon matched-pairs tests assigning males to categories of 'winners' and 'losers', depending on which of the two obtained more eggs.

Paired *t*-tests were applied to see if body size differed between the males of the two species used in each experiment. We also performed paired *t*-test on the body size of winners and losers to test if size had an influence on mate choice, regardless of species identity.

To quantify the degree of premating isolation between any two groups, isolation indices were calculated using $I_{\text{trial}} = (E_{\text{con}} - E_{\text{hetero}})/E_{\text{total}}$ where $E_{\text{con}} - E_{\text{hetero}}$ are the number of eggs spawned with con- and heterospecific males in a trial and E_{total} is the total number of eggs in that trial. *I* can range from −1 to 1, with negative values indicating a preference for heterospecific males and positive values indicating a preference for conspecifics. A value of zero would indicate no preference. Results were averaged over trials within experiment, to obtain a measure of isolation for each of the two female types tested ($I_{\text{exp1–4\_female type}}$). This was necessary to detect asymmetry in assortative mating depending on which female type was asked to choose between con- and heterospecific males.

To obtain an overall isolation index for each of the four group comparisons ($I_{\text{exp1–4}}$), we then averaged over the two female type-specific isolation indices.

Analysis of genetic distance

To compare genetic distances between the four groups used in our mate choice trials, we calculated pairwise genetic distances as uncorrected p-distances from mitochondrial DNA sequences (D-loop) of between 4 and 14 individuals per group (Mendelson, 2003; Chapman & Burke, 2007). All distances were corrected for within-group variation among haplotypes by subtracting mean within-group distances from mean pairwise between-group distances (Nei, 1987; Mendelson, 2003) (Table 1b).

	philander Bangweulu	*philander* Mweru	*weed picker* Mweru	*grey back* Mweru
(a) Premating isolation				
philander Bangweulu	–	0.061	–	–
philander Mweru		–	0.227	0.404
weed picker Mweru			–	0.401
grey back Mweru				–
(b) Genetic distance				
philander Bangweulu (12)	–	0.0268	0.024	0.0257
philander Mweru (4)		–	0.0015	0.0002
weed picker Mweru (10)			–	0.0009
grey back Mweru (7)				–
(c) Phenotypic distance (ecomorphology)				
philander Bangweulu (16)	–	0.369	0.347	0.382
philander Mweru (15)		–	0.408	0.434
weed picker Mweru (13)			–	0.445
grey back Mweru (18)				–
(d) Phenotypic distance (male colouration)				
philander Bangweulu (7)	–	0.279	0.236	0.546
philander Mweru (6)		–	0.346	0.391
weed picker Mweru (13)			–	0.607
grey back Mweru (8)				–

The number of individuals per group is shown in brackets.

Table 1 (a) Premating isolation indices for all four groups tested in mate choice experiments. (b) Pairwise genetic distance calculated as uncorrected p-distances from DNA sequences of the mitochondrial D-loop region. (c) Phenotypic distance (P_{st}) calculated from PC1 scores of 13 ecomorphological measurements. (d) Phenotypic distance (simple mismatch coefficients) calculated from male colouration scores at 10 morphological landmarks.

Analysis of ecomorphological distance

To quantify ecomorphological variation within and among the four groups, we calculated phenotypic distances from 13 linear morphological characters measured with digital callipers to the nearest 0.01 mm on between 13 and 18 individuals per groups (Table 1c). All morphological measures were taken on wild fish. The traits measured were: standard length (SL), body depth (BD), head length (HL), head width (HW), snout length (SnL), snout width (SnW), lower jaw length (LjL), lower jaw width (LjW), eye length (EyL), eye depth (EyD), interorbital width (IoW), cheek depth (ChD) and preorbital depth (PoD). Measuring differences in morphological characters is a standard technique to detect differences in resource use and has been shown to reflect adaptation to different habitats in fishes and cichlids in particular (Caldecutt & Adams, 1998; Ruber & Adams, 2001; Hendry *et al.*, 2002; Kassam *et al.*, 2003; Clabaut *et al.*, 2007).

Raw measurements were log-transformed prior to all analyses to homogenize variance. Normal distributions for all traits were confirmed with Shapiro-Wilk tests. There were significant differences in standard length between groups (one-way ANOVA: $F_{3,61} = 2.99$, $P = 0.038$). A Tukey-Kramer test showed that *weed picker* were on average longer (77.45 cm) than all other species and significantly differed from *grey backs* (64.65 cm). *Philander* Mweru (71.58 cm) and *philander* Bangweulu (68.38 cm) were intermediate in size.

Because of the size differences between types, we applied statistical corrections following methods described in Hendry *et al.* (2000). First, linear regressions were calculated within groups for each of the traits against either SL (in case of BD, HL) or HL (in case of the remaining nine traits). These group-specific slopes (b) were then used to standardize each measurement (M_{std}) by the observed standard length of each fish (L_o) divided by the mean standard or head length (L_x) of all four groups pooled using the expression $M_{std} = M_o(L_o/L_x)^b$. The adjusted trait values were entered into a principal component analysis (PCA). One-way ANOVAS were run both on standardized traits (M_{std}) separately and on the first three principle components to detect between-groups differences. Tukey-Kramer tests were applied for pairwise population comparisons.

Canonical linear discriminant analysis (Ostbye *et al.*, 2005) was used to explore if individuals could be correctly reassigned to their source group based on the adjusted, morphological traits (M_{std}) alone. This method calculates the distance of each individual to the multivariate group mean (Mahalanobis distance), which was visualized by plotting canonical axis 1 and 2 (Fig. 5). It also allowed for ranking the 13 traits based on their discriminating power using a stepwise forward variable selection model where the highest ranking trait was entered first.

The divergence in quantitative phenotypic traits between groups was estimated by calculating P_{st} values [the phenotypic surrogate for Qst (Spitze, 1993)] for each group comparison. We calculated P_{st} for each of the first three principal components of the size adjusted morphological data. We then calculated a weighted average over all three components, using the amount of variance explained by each component as weight, to obtain a total measure of phenotypic divergence on the major axes of shape variation.

Analysis of male nuptial colouration

To quantify variation in male nuptial colouration within and among groups, we visually scored the colour of males in breeding dress at 10 different places on body and fins (Fig. 1c). All measurements were taken on wild fish. At every landmark, colour was scored as either translucent, white, grey, black, blue, green, yellow, orange or red. Scoring was done by one individual (OS) without knowledge of the source population of the fish. To convert the qualitative score data into a distance metric, we used the 'simple matching coefficient' (SMC) (Digby & Kempton, 1987) dividing the number of matching characters by the total number of characters in individual pairwise comparisons. We collected data from photographs of between 6 and 13 different wild caught males per group (Table 1d). All colour distances were corrected for within-group variation by subtracting the average intrapopulation distance from the average interpopulation distance. Canonical linear discriminant analysis was used to explore if individuals could be correctly reassigned to their source population based on their colour scores alone.

Testing genetic and phenotypic distance as predictors of premating isolation

To test the effects of genetic and phenotypic distance on the degree of premating isolation, we regressed premating isolation (I_{exp1-4}) against genetic distance (uncorrected p-distance), ecomorphological distance (P_{st}) and colouration distance (SMC) (Fig. 7) using either linear (morphology and genetic distance) or logarithmic (colouration) regression models. Normality of distribution was tested with Shapiro-Wilk tests and genetic distance data was log-transformed to acquire normality.

We predicted that if phenotypic differentiation promoted premating isolation, then significant assortative mating would be observed in experiments 1–3 (genetically closely related but phenotypically divergent species from same lake). To test this, we performed a two-factorial ANOVA with egg number per male as dependent variable. The factors were 'female type' (fixed with two levels), 'male type' (fixed with two levels) and the interaction term between them (female type × male

type). With the null hypothesis of random mating, our prediction of assortative mating between the three ecologically different members of the Lake Mweru radiation would be confirmed by a significant interaction term between male and female type.

In Experiment 4, we tested males and females representing distantly related populations of similar phenotype but from different lakes (i.e. different 'origins'). To compare the importance of phenotypic divergence vs. genetic distance, we performed a two-factorial ANOVA on egg number with the factors 'female origin' and 'male origin' (either Mweru or Bangweulu) and the interaction term between them (female origin × male origin). Here, the null hypothesis assumes assortative mating, hence the nonsignificance of the interaction term would support our prediction, that 'origin' has no impact on the strength of premating isolation.

Lastly, we tested on results pooled across all four experiments if the interaction between female and male type term remained significant when genetic distance (uncorrected p-distance) was added to the model (testing 'female type', 'male type', the interaction term between them and genetic distance on the number of eggs). All analyses were performed in JMP PROFESSIONAL 6.0.0 (SAS Institute, Cary, NC, USA).

Results

Mate choice experiments

In Experiment 1, 10 spawnings were obtained from each group (*grey back*, *philander* Mweru; Fig. 4a). *Philander* Mweru females spawned on average 19.5 ± 21 eggs with *philander* Mweru males and only 3.2 ± 5.1 eggs with *grey back* males. *Grey back* females spawned 16.8 ± 25.7 eggs with *grey back* males and 7.9 ± 13 eggs with *philander* Mweru males. *Philander* Mweru females preferred their own male type ($t_9 = 2.14$, $P = 0.031$; all *P*-values one-tailed, Fig. 4a). *Grey back* females on the other hand did not mate significantly assortatively ($t_9 = -0.86$, $P = 0.205$). Overall, there was significant assortative mating in this species pair ($t_{19} = -2.0$, $P = 0.030$). This pattern was confirmed when ranking the paired males of each trial by the number of eggs they obtained (categorizing males as either 'winners' or 'losers'). With *philander* females, *philander* males obtained significantly more often a higher number of eggs than *grey back* males (Wilcoxon matched-pairs test (all *P*-values one-tailed): $Z = -1.99$, $P = 0.024$). There was no such effect when ranking males in trials with *grey back* females ($Z = -0.97$, $P = 0.166$).

In Experiment 2, 13 spawnings were obtained from each group (*weed picker*, *philander* Mweru; Fig. 4b). *Philander* Mweru females spawned on average 36.8 ± 23.3 eggs with *philander* Mweru males and only 5.2 ± 9.6 eggs with *weed picker* males. *Weed picker* females spawned 16.8 ± 25.4 eggs with *weed picker* males and 23.4 ± 19.1 eggs with *philander* Mweru males. While *weed picker*

females did not mate assortatively ($t_{12} = -0.58$, $P = 0.285$; Fig. 4b), *philander* Mweru females again mated strongly assortatively ($t_{12} = -3.89$, $P = 0.001$). Overall, there was a trend for assortative mating in this species pair ($t_{25} = 1.59$, $P = 0.061$). Again, this pattern was confirmed when ranking the paired males of each trial by the number of eggs they obtained. With *philander* Mweru females, *philander* Mweru males obtained significantly more often a higher number of eggs than *weed picker* males ($Z = -2.69$, $P = 0.004$). There was no such effect when ranking males in trials with *weed picker* females ($Z = -0.91$, $P = 0.176$).

In Experiment 3, six spawnings were obtained from *grey back* females and five spawnings from *weed picker* females (Fig. 4c). *Grey back* females spawned on average 17.8 ± 15.4 eggs with *grey back* males and only 2.3 ± 3.01 eggs with *weed picker* males. Conversely, *weed picker* females spawned 23 ± 20.8 eggs with *weed picker* males and only 4 ± 6.5 eggs with *grey back* males. Significant assortative mating was found for *grey back* females ($t_5 = 2.14$, $P = 0.043$) and a assortative trend was observed for *weed picker* females ($t_4 = -1.69$, $P = 0.055$; Fig. 4c). Overall, there was significant assortative mating in this species pair ($t_{10} = -2.79$, $P = 0.010$). Ranking the males by the number of eggs obtained yielded a strong trend for *grey back* males to be more successful with *grey back* females ($Z = -1.57$, $P = 0.055$) whereas there was no trend for *weed picker* males to be more successful with *weed picker* females ($Z = -1.22$, $P = 0.116$).

In Experiment 4, 10 spawnings were obtained from each group (*philander* Bangweulu, *philander* Mweru; Fig. 4d). Bangweulu females spawned on average 17.3 ± 16.6 eggs with Bangweulu males and 5.5 ± 7.5 eggs with Mweru males. Mweru females spawned 6.5 ± 9.6 eggs with Mweru males but 9.3 ± 8.8 eggs with Bangweulu males. No assortative mating was found for either of the female types (*philander* Bangweulu females: $t_9 = 1.7$, $P = 0.061$; *philander* Mweru females: $t_9 = 0.58$, $P = 0.29$; Fig. 4d). The two male types were not ranked significantly differently when competing for Bangweulu females ($Z = -1.58$, $P = 0.072$) or for Mweru females ($Z = -1.01$, $P = 0.155$).

Premating isolation index

Testing for assortative mating between the ancestral *philander* phenotype from Lake Mweru and the sympatric derived *grey back* (Experiment 1) yielded similar premating isolation indices for both female types ($I_{exp1\_philander\ Mweru} = 0.377$; $I_{exp1\_grey\ back} = 0.43$, positive values indicate assortativeness), resulting in the highest overall isolation index observed in this study ($I_{exp1} = 0.404$).

The same test revealed assymmetric isolation between the ancestral phenotype and the other derived sympatric type, *weed picker* (Experiment 2). While *philander* Mweru females clearly preferred their own type, *weed picker* females did not ($I_{exp2\_philander\ Mweru} = 0.228$;
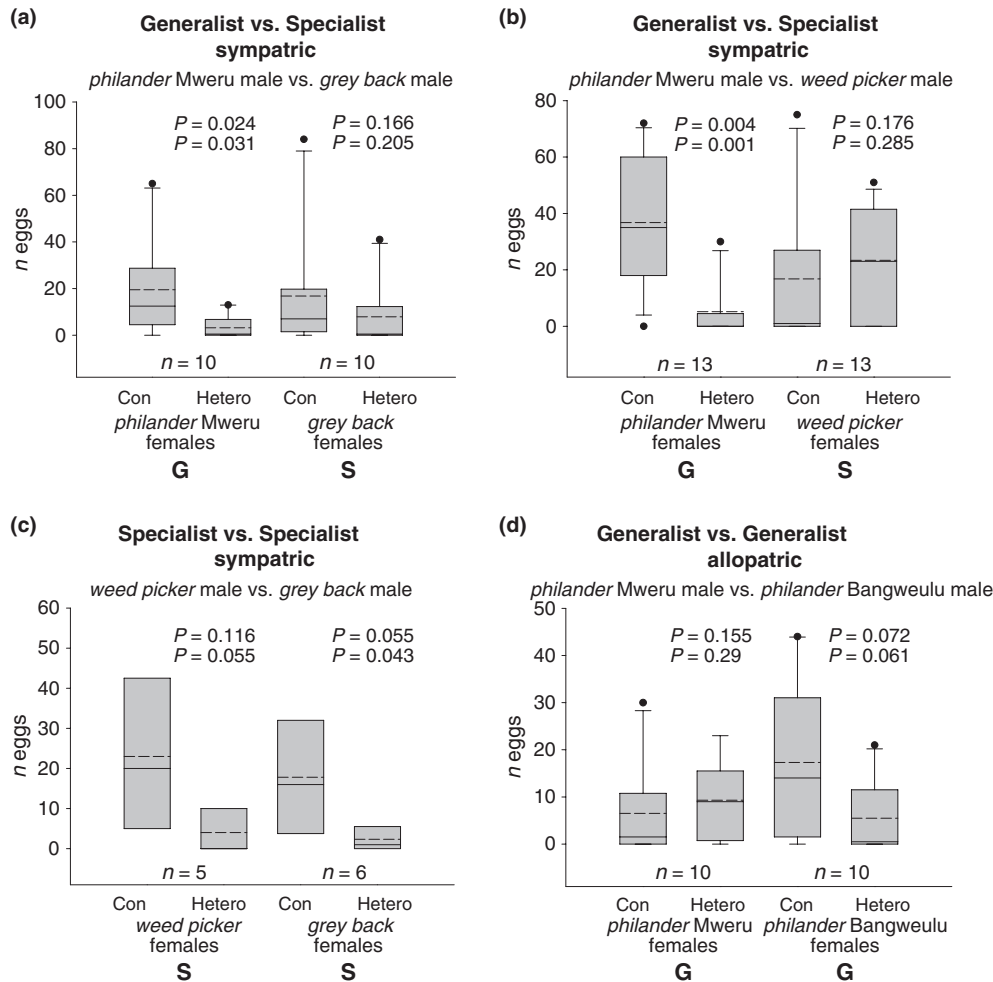
Fig. 4 Results of mate choice experiments between four groups of *Pseudocrenilabrus* with varying degrees of genetic and phenotypic divergence. *Philander* Mweru females mated assortatively against both closely related, sympatric types (a, b), but not against males from the more distantly related, phenotypically similar allopatric population (d). The two genetically similar, ecologically derived species mated randomly when offered their own and the sympatric generalist species (a, b), but assortatively when tested against each other (c). The letters G and S stand for 'generalist' and 'specialist'. Dashed lines show the average number of eggs spawned with either conspecific ('con') or heterospecific ('hetero') males. Solid lines show the median number of eggs. Boxes indicate upper and lower quartiles. Whiskers extend to the 5th and 95th percentiles. Outliers are shown as dots. Upper *P*-values are from Wilcoxon ranked-pairs tests (one-sided), the lower are *P*-values (one-sided) from paired *t*-tests comparing the number of eggs spawned with con- and heterospecific males per female type. Sample sizes are given per female type.

$I_{exp2\_weed\ picker} = -0.237$), resulting in weaker overall premating isolation for this species pair ($I_{exp2} = 0.227$).

Both females of the two sympatric derived types from Lake Mweru (Experiment 3) mated assortatively ($I_{exp3\_grey\ back} = 0.442$; $I_{exp3\_weed\ picker} = 0.36$), resulting in a high overall isolation index ($I_{exp3} = 0.401$).

When *philander* females from Lakes Bangweulu and Mweru were choosing between males of their own population vs. the distantly related allopatric population (Experiment 4), only Bangweulu females preferred males of their own population ($I_{exp4\_philander\ Bangweulu} = 0.368$), while Lake Mweru females did not ($I_{exp4\_philander\ Mweru} = -0.246$), yielding a low overall premating isolation index

($I_{exp4} = 0.061$). All premating isolation indices are summarized in Table 1a.

The effect of body size on mate choice

Male body size had no effect on female preference. Winners and losers did not differ in body size in any of the experiments. There was one exception to this: in Experiment 4, *philander* Mweru females chose significantly more often to lay more eggs with the bigger male [$t = 2.33$, P (one-tailed) $= 0.022$]. At the same time, the two different male types (*philander* Mweru and *philander* Bangweulu) did not differ significantly in body size.

Conversely, in Experiment 2 with *weed picker* females, significant differences in body size were found between *weed picker* (average size = 7.17 ± 0.86 cm) and *philander* Mweru males (average size = 7.75 ± 0.74 cm), but when assigning the males of this experiment to the winner and the loser category, no difference in body size was detected [$t = 0.99$, P (one-tailed) = 0.17]. W*eed picker* females hence did not generally prefer to mate with the larger male, regardless of species identity. Likewise, in Experiment 1, *philander* males (average size = 5.64 ± 1.1 cm) were larger than grey back males (average size = 4.97 ± 1.33 cm), but *philander* Mweru females did not preferentially mate with the larger male [$t = -0.38$, P (one-tailed) = 0.37]. In Experiment 3, no size differences were detected between *grey back* and *weed picker* males, and female mate choice was not affected by size.

Genetic divergence

Pairwise genetic distances found between allopatric populations were 30 times greater than the genetic distance between the three sympatric species from Lake Mweru (distances between all four populations are summarized in Table 1b).

Ecomorphological divergence

Significant between-group differences were detected for each of the body size-adjusted morphological traits in one-way ANOVAS, except for head length, snout length, cheek depth and preorbital depth (all other $P < 0.05$; morphological raw data for each of the 13 traits with mean and SD per group is provided as Supporting Information (Table S1). Tukey-Kramer tests showed that the allopatric *philander* Bangweulu and *philander* Mweru populations did not significantly differ in any trait except for eye depth. This is consistent with the prediction that the two populations indeed have similar morphological adaptations and inhabit similar ecological niches in the two different lakes although more direct tests of niche overlap would be required to confirm this.

Grey backs were significantly more elongate (smaller body depth) and had narrower snouts than all other groups. They also had a shorter but wider lower jaw, and larger eyes than all other groups. This may reflect their putatively demersal foraging mode, feeding on detritus and insect larvae in the offshore demersal zone of the lake.

Weed pickers were best characterized by their head width, which was significantly larger than in any of the other groups. Further, they had the longest lower jaws and snouts of all, consistent with feeding on insect larvae and other benthic animals in the littoral vegetation zone.

When performing a PCA on all standardized traits [without standard length because it was already incorporated into the calculation of (M_{std})], the first three PCs

explained 49%, 15% and 11%, respectively, of the variance in the data set (Fig. S1). There were significant differences between groups, not along PC1, but along PC2 and 3 (PC2: $F_{3,61} = 23.71$, $P < 0.001$; PC3: $F_{3,61} = 10.41$, $P < 0.001$). This method separated *grey back*s from the three other types along PC2. *Grey back*s occupy an area in morphospace that does not overlap with that of the other two sympatric species, and slightly overlaps with that of the allopatric population. The morphospace of *weed picker* shows considerable overlap with that of *philander* Mweru and *philander* Bangweulu along PC1 and 2, but was different from sympatric *philander* Mweru along PC3. On the contrary, *philander* Bangweulu and *philander* Mweru broadly overlapped in morphospace along all three axes. *Philander* Bangweulu showed some sub-grouping along PC1.

The canonical discriminant analysis correctly assigned all but seven out of 62 individuals to the four source groups (Pillai's trace = 7.62; $P < 0.001$; Fig. 5). The seven mis-assignments all involved *philander* Bangweulu, but there was no wrong assignment among the three sympatric species from Lake Mweru. After applying stepwise forward selection of variables, five traits significantly contributed to the model (BD, HL, HW, LjL and ChD). The first, second and third canonical axes explained 62%, 26% and 12% of the variance in the data set.

Strongest phenotypic differentiation was found between the two ecologically derived specialists from Lake Mweru, *weed picker* and *grey back* ($P_{st} = 0.445$, all P_{st}-test results summarized in Table 1c). This was followed by the phenotypic distances between the
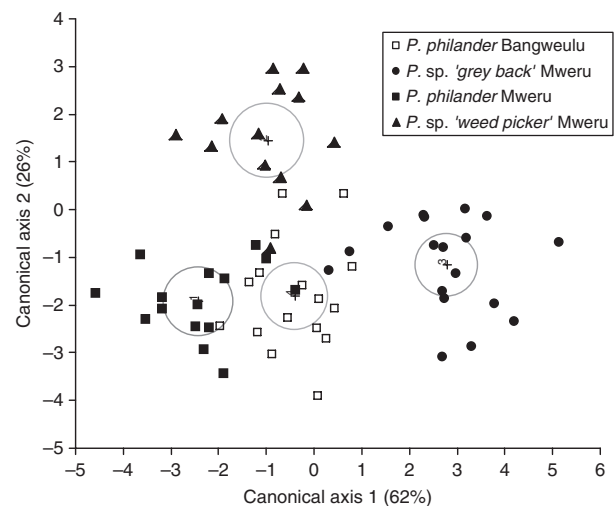

Fig. 5 Canonical discriminate analysis based on 13 morphological traits measured on individuals from the four different groups. All but seven individuals were assigned correctly to their source group. Circles represent 95% confidence interval spheres around multivariate group mean (indicated by +).

sympatric generalist, *philander* Mweru and both derived specialists (*philander* Mweru vs. *grey back* Mweru: $P_{st} = 0.434$; *philander* Mweru vs. *weed picker* Mweru: $P_{st} = 0.408$). The allopatric generalist, *philander* Bangweulu was moderately different from one of the specialists from Lake Mweru (*philander* Bangweulu vs. *grey back* Mweru; $P_{st} = 0.382$). The divergence found between *philander* Mweru and *philander* Bangweulu was comparatively small ($P_{st} = 0.369$). Only the comparison between *philander* Bangweulu and *weed picker* Mweru revealed even more phenotypic similarity ($P_{st} = 0.347$).

In summary, largest distances in ecomorphology were found among the three sympatric Lake Mweru species, while the allopatric *philander* Lake Bangweulu was similar to two members (*philander* and *weed picker*) of the Lake Mweru radiation.

Divergence in male colouration

Canonical discriminant analysis revealed that the three sympatric species from Lake Mweru have completely nonoverlapping male nuptial colouration (Fig. 6). At the same time, *philander* Bangweulu and *philander* Mweru were relatively similar in colouration, and *philander* Bangweulu was intermediate between the two Lake Mweru species *philander* and *weed picker*. All but one of 35 individuals were correctly assigned to the four source groups (Pillai's trace = 4.49; $P < 0.001$). The one exception was a *philander* Bangweulu which was more similar to *weed pickers*. After applying stepwise forward selection of variables, three traits significantly contributed to the model (colouration of dorsal fin, flanks and anal fin). The first, second and third canonical axes explained 95%, 4% and 0.5% of the variance in the data set.

Using metric distances calculated as SMC, highest dissimilarity in colouration was found between the two sympatric ecological specialists from Lake Mweru, *weed picker* and *grey back* (SMC = 0.607; all SMC-test results are summarized in Table 1d). Colour differences between
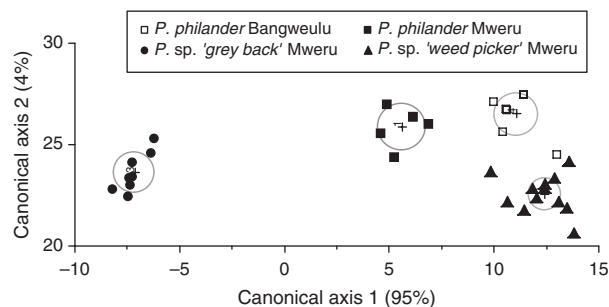
the two ecologically derived species and the two generalists were small to moderately strong (*philander* Bangweulu vs. *grey back*: SMC = 0.546; *philander* Bangweulu vs. *weed picker*: SMC = 0.236; *philander* Mweru vs. *grey back*: SMC = 0.391; *philander* Mweru vs. *weed picker*: SMC = 0.346). The two populations of ancestral phenotype *philander* Mweru and *philander* Bangweulu were rather similar in colouration (SMC = 0.279). As with ecomorphology, the similarity between *philander* Bangweulu and *weed picker* was slightly surprising, given our prediction that the two generalists from the different lakes would show the highest phenotypic match.

However, as expected largest dissimilarities in colour were found among the three sympatric species from Lake Mweru, whereas the allopatric *philander* populations were rather similar in phenotype. Remarkably, the dissimilarity patterns in male colour between the four groups closely resembled those in ecomorphology.

Predictors of premating isolation

Phenotypic distance was a good predictor of premating isolation (Fig. 7). The greater the ecomorphological distance between groups, the stronger was assortative mating between them (linear regression: $R^2 = 0.96$, $F_{1,4} = 79.75$, $P = 0.012$). The same pattern, but with a weaker effect on premating isolation, was observed for male colouration. Assortative mating increased with increasing differences in colouration but the relationship
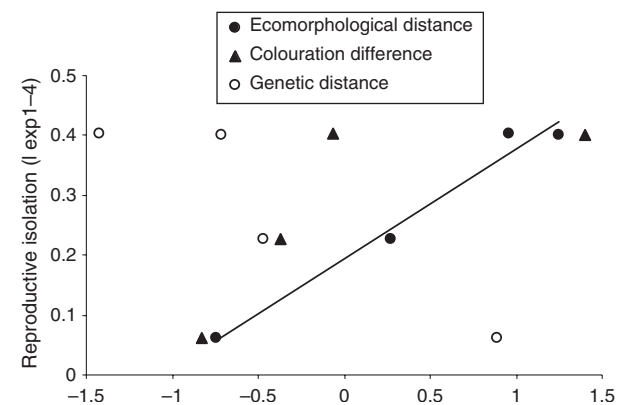
Fig. 7 Testing genetic and phenotypic distance as predictors of premating isolation (represented by the isolation index I_{exp1-4}) between the four groups. Isolation increased with ecomorphological distance (significantly, regression line shown) and with colouration differences (non-significantly), but not with genetic distance. Genetic distance was calculated as pairwise uncorrected p-distances from mitochondrial D-loop sequences. Ecomorphological distance (P_{st}) for the same groups were calculated from 13 morphological measurements. Male colouration differences (simple mismatch coefficients) were calculated from male colouration scores at 10 morphological landmarks. The *x*-axis was standardized for better comparison.

Fig. 6 Canonical discriminate analysis based on male colouration scores taken on 10 morphological landmarks. All but one individual were assigned correctly to their source group. Circles represent 95% confidence interval spheres around multivariate group mean (indicated by +).

was not significant (logarithmic regression: $R^2 = 0.69$, $F_{1,4} = 4.37$, $P = 0.172$). The slope of the curve of premating isolation against genetic distance, in contrast, was negative such that at larger genetic distances mating tended to be more random (linear regression: $R^2 = 0.87$, $F_{1,4} = 13.52$, $P = 0.067$).

When females were allowed to choose between males of their own species and males of a phenotypically different, but closely related species from the same lake (Experiments 1–3) the interaction between male and female species was significant ($R^2 = 0.14$, $F_{3,114} = 7.33$, P (model) < 0.001, P (interaction) < 0.001), confirming our prediction of assortative mating by phenotype. When females were given the choice between males of their own population and males of a phenotypically similar but distantly related population from a different lake (experiment 4) the interaction term between male and female origin remained non-significant ($R^2 = 0.09$, $F_{3,39} = 2.27$, P (model) = 0.10, P (interaction) = 0.21) confirming our prediction that population origin (Lake Mweru or Bangweulu) did not play a role in mating decisions.

Genetic distance between groups did not predict the number of eggs laid when 'species identity' (i.e. the interaction term between female and male type) was left out of the model (on results of all four experiments: $R^2 = 0.02$, $F_{1,153} = 3.38$, $P = 0.07$), again confirming that the amount of genetic differentiation per se had no effect on mate choice. When using both parameters, genetic distance and species identity, in a combined model on data from all four experiments to compare their respective effect sizes, species identity clearly exceeded the effect of genetic distance on premating isolation ($R^2 = 0.14$, $F_{1,153} = 7.18$, P (model) < 0.001, P (identity) < 0.001, P (genetic distance) = 0.10).

Discussion

One of the outstanding questions in our understanding of adaptive radiations is, which sources of selection initiate the diversification of species and how can assortative mating evolve in the face of gene flow. An idea that emerged early in the literature (Simpson, 1944) is that the access to new ecological resources can promote population divergence in early stages of adaptive radiation. This idea has received considerable empirical and theoretical support (Schluter, 2000; Rundle & Nosil, 2005). Many species in young adaptive radiations are genetically isolated by divergent mating preferences. Yet, whether the emergence of such premating barriers to gene flow typically can occur without episodes of geographical isolation between the diverging populations is still controversial, not least because empirical evidence from the wild is scarce.

The hypothesis of ecological speciation (Schluter, 2001) argues that assortative mating can evolve along with ecological adaptations to different environments,

either because traits under divergent natural selection are linked to traits affecting mate choice (e.g. (Rundle *et al.*, 2000; Vines & Schluter, 2006), or because sexual selection regimes differ between different ecological niches, inducing shifts in mate choice criteria between alternative traits or trait states (e.g. Boughman, 2002; Maan *et al.*, 2006; Seehausen *et al.*, 2008). Because the mechanisms of ecological speciation are thought to potentially work, regardless of the geographical scenario, the ecological speciation hypothesis is particularly interesting when trying to explain speciation in young adaptive radiations, i.e. after the colonization of a new habitat without strong *a priori* barriers to gene flow.

Here, we tested one prediction of the ecological speciation hypothesis (McPeek & Wellborn, 1998) that phenotypic rather than genetic distance explains the strength of assortative mating among members of a young radiation of cichlid fish of the genus *Pseudocrenilabrus* in Lake Mweru (Zambia/DRC). Three of the species tested in mate choice experiments represent genetically closely related, but ecologically and phenotypically divergent sympatric species. The first is the widely occurring and formally described *P. philander*, a generalist species that represents the ancestral phenotype in the Lake Mweru radiation. The second, *Pseudocrenilabrus* sp. *weed picker*, inhabits the densely vegetated littoral zone and is an ecologically derived specialist, feeding on insect larvae. The third, *Pseudocrenilabrus* sp. *grey back*, is an offshore demersal feeder representing another derived, highly specialized ecotype. Further, we tested *P. philander* from Lake Mweru against an allopatric population of *P. philander* from Lake Bangweulu (Zambia) which is genetically distant, but phenotypically and ecologically very close to *P. philander* from Lake Mweru.

We found that (1) females of the ancestral, generalist species from Lake Mweru mated assortatively against both of the sympatric, genetically closely related but phenotypically distinct species; (2) females of the two closely related but phenotypically distinct species from Lake Mweru preferred their conspecific males over males of the other derived species, but had no strong preferences for their own males over males of the ancestral phenotype; (3) females of the allopatric, genetically distant, but phenotypically similar populations of *P. philander* mated randomly with each other (Fig. 4). We found all three sympatric species highly differentiated in both ecomorphological traits and male breeding colouration (Figs 5 and 6). At the same time, the two allopatric, generalist populations were similar to each other in both colour and morphology, confirming our prediction of niche conservatism, i.e. the tendency that both have retained ancestral ecological characteristics associated with *P. philander* over much of its geographical range. Interestingly, the allopatric population of *P. philander* from Lake Bangweulu, though similar to

P. philander from Lake Mweru in morphospace and colouration, was intermediate between all three Lake Mweru species, suggesting character displacement associated with speciation in Lake Mweru. The sub-grouping in morphospace observed within Bangweulu *P. philander* (Fig. S1) suggests that the ancestral population not only consisted of uniform generalist phenotypes but instead showed considerable phenotypic diversity which could be recruited for niche divergence during radiation.

When assessing different predictors of premating isolation, only phenotypic distance but not genetic distance explained the variance in strength of assortative mating (Fig. 7). Larger ecomorphological and male breeding colour differences were associated with larger premating isolation, while differences in ecomorphology and in male colouration were significantly positively correlated (Spearman's rank correlation: $R_s = 0.83$, $P = 0.042$). These results are consistent with ecological speciation. However, it must be acknowledged at this point that the relationship between morphological differences and the ecological diversification of the populations studied here remains untested. Additional analyses of the functional morphology are necessary to test the adaptive relevance of body form variation of these fishes in different environmental niches. Also, for a conclusive test of our assumption that Lake Mweru's phenotypic diversity and the differences in mating preferences have evolved because of ecologically divergent selection, the heritability of traits relative to the variation caused by developmentally plastic responses to inhabiting a particular habitat needs to be assessed. Another point to consider is that, unlike the sympatric comparison, the allopatric population comparison was only replicated once here. If other ecologically similar allopatric populations mated assortatively, our conclusion that random mating was due to an absence of ecological speciation between allopatric populations would have to be revaluated. Hence, this study would benefit from further replication of the allopatric comparison.

Although we do not know the phenotypic traits that female *Pseudocrenilabrus* from Lake Mweru use in mate choice, the correlation between ecomorphological and nuptial colour divergence suggests an important role of ecological selection in the origin of divergent mating preferences. Variation in nuptial colour may become associated with variation in traits relevant to resource utilization either through ecological reinforcement where colour becomes a marker of ecological variation (Gavrilets, 2004; Reynolds & Fitzpatrick, 2007; Jiggins *et al.*, 2008) or through habitat-dependent sexual selection (Boughman, 2002; Seehausen *et al.*, 2008). Although colour divergence in the *Pseudocrenilabrus* incipient species is significant, it is generally more muted than in other cichlid radiations (e.g. compared with Lake Victoria) and exhibits variations on the same colour theme rather than major shifts in hue.

Our data suggests that mating preferences can diverge rapidly when associated with ecological divergence during adaptive radiation despite the absence of geographical isolation. At the same time our data suggests that allopatric populations, living in similar environments and maintaining ancestral phenotypes, may fail to evolve premating isolation even after very long periods of geographical isolation. Relaxed molecular clocks (Genner *et al.*, 2007) applied to our sequence data suggest that the population of *P. philander* from Lake Bangweulu has been isolated from the populations in Lake Mweru for between 1 570 000 and 2 780 000 years (Stelkens *et al.* in prep.). Such genetically ancient but phenotypically conserved populations are likely to hybridize should they experience secondary contact.

One of the derived types (*grey back*) shows a significantly smaller genetic distance ($t = 5.66$, $P < 0.01$) to the sympatric *philander* Mweru than the other derived type (*weed picker*) to *philander* Mweru (Table 1b). Interestingly, the strength of assortative mating we found in the genetically less distant pair (*grey back* vs. *philander* Mweru) was almost two-fold stronger than in the genetically more distant pair (*weed picker* vs. *philander* Mweru; Table 1a). At the same time, larger phenotypic differences in both morphology and colouration were found between *grey back* and *philander* Mweru. This may provide additional support for the hypothesis that divergent ecological selection, and not divergence time, predicts assortative mating between the young species in the radiation.

Hybrids between all three Lake Mweru species were fully viable when raised in a laboratory environment (R. Stelkens, unpublished data) and there is no evidence for strong intrinsic post-zygotic isolation barriers among species in other cichlid radiations with comparably recent divergence times (Crapon de Caprona & Fritzsch, 1984; Seehausen *et al.*, 1997; van der Sluijs *et al.*, 2008). This makes it unlikely that reinforcement through endogenous fitness disadvantages contributed to the evolution of premating incompatibilities in the *Pseudocrenilabrus* of Lake Mweru. We have not yet tested for ecological reinforcement-like mechanisms in this system and its role in driving the evolution of mate choice, but other work suggests this could be important in cichlid fish speciation (Seehausen *et al.*, 2008). A possible experiment to isolate effects of ecological by-product mechanisms vs. ecological reinforcement would involve mate choice trials between ecologically divergent populations that have no history of sympatry (Hollander *et al.*, 2005; Vines & Schluter, 2006). If for instance, *P. philander* Bangweulu females mated at random when choosing between males of their own species and males of the phenotypically divergent species from Lake Mweru (e.g. *grey back*), we could conclude that ecologically based reinforcement was involved in speciation in this system. These experiments are currently in planning.

Pseudocrenilabrus is known as a genus with a panafrican distribution, yet with very low species numbers. Only three formally described species exist, and some ten geographically fully isolated subspecies of *P. philander* are thought to be the result of classical allopatric divergence between river basins (Katongo *et al.*, 2005). This failure to undergo adaptive radiations despite presence in various large lakes has been puzzling (Seehausen, 2006), but had sometimes been explained by habitat conservatism with the riverine and lake margin habitats typical of *Pseudocrenilabrus* thought to not provide ecological opportunity for the evolution of divergent adaptations (Schluter, 2000); but see (Koblmueller *et al.*, 2008). Our data here show that if given the ecological opportunities of a heterogeneous lake, *Pseudocrenilabrus* may radiate quickly into multiple species similar to some other groups of cichlid fish. We now need to explain why *Pseudocrenilabrus* radiated in Lake Mweru but not in any of the other large lakes in the geographical range of the genus.

In summary, we found that phenotypic differences, including eco-morphology and male nuptial colouration, but not genetic distance, promotes assortative mating in the adaptive radiation of sympatric *Pseudocrenilabrus* cichlids in Lake Mweru. Our data provide support for ecological speciation in the face of gene flow.

Acknowledgments

References

Amorim, M.C.P., Knight, M.E., Stratoudakis, Y. & Turner, G.F. 2004. Differences in sounds made by courting males of three closely related Lake Malawi cichlid species. *J. Fish Biol.* **65**: 1358–1371.

Baldwin, B.G. 2007. Adaptive radiation of shrubby tarweeds (Deinandra) in the Californian Islands parallels diversification of the Hawaiian silversword alliance (Compositae-Madiinae). *Am. J. Bot.* **94**: 237–248.

Boughman, J.W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* **411**: 944–948.

Boughman, J.W. 2002. How sensory drive can promote speciation. *Trends Ecol. Evol.* **17**: 571–577.

Caillaud, M.C. & Via, S. 2000. Specialized feeding behavior influences both ecological specialization and assortative mating in sympatric host races of pea aphids. *Am. Nat.* **156**: 606–621.

Caldecutt, W.J. & Adams, D.C. 1998. Morphometrics of trophic osteology in the threespine stickleback, *Gasterosteus aculeatus*. *Copeia* **4**: 827–838.

Chapman, M.A. & Burke, J.M. 2007. Genetic divergence and hybrid speciation. *Evolution* **61**: 1773–1780.

Clabaut, C., Bunje, P.M.E., Salzburger, W. & Meyer, A. 2007. Geometric morphometric analyses provide evidence for the adaptive character of the Tanganyikan cichlid fish radiations. *Evolution* **61**: 560–578.

Coyne, J.A. & Orr, H.A. 2004. *Speciation*, Sinauer Associates, Sunderland, MA.

Crapon de Caprona, M.-D. & Fritzsch, B. 1984. Interspecific fertile hybrids of haplochromine cichlidae (Teleostei) and their possible importance for speciation. *Neth. J. Zool.* **34**: 503–538.

Digby, P.G.N. & Kempton, R.A. 1987. *Multivariate Analysis of Ecological Communities*. Chapman and Hall, London.

Dobzhansky, T. 1951. *Genetics and the Origin of Species*. Columbia University Press, New York.

Dodd, D.M.B. 1989. Reproductive isolation as a consequence of adaptive divergence in *Drosophila-Pseudoobscura*. *Evolution* **43**: 1308–1311.

Doebeli, M. & Dieckmann, U. 2003. Speciation along environmental gradients. *Nature* **421**: 259–264.

Endler, J.A. 1992. Signals, signal conditions, and the direction of evolution. *Am. Nat.* **139**: S125–S153.

Friar, E.A., Prince, L.M., Roalson, E.H., McGlaughlin, M.E., Cruse-Sanders, J.M., De Groot, S.J. & Porter, J.M. 2006. Ecological speciation in the East Maui-endemic Dubautia (Asteraceae) species. *Evolution* **60**: 1777–1792.

Funk, D.J. 1998. Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. *Evolution* **52**: 1744–1759.

Funk, D.J., Nosil, P. & Etges, W.J. 2006. Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proc. Natl. Acad. Sci. USA* **103**: 3209–3213.

Gavrilets, S. 2003. Perspective: models of speciation: what have we learned in 40 years? *Evolution* **57**: 2197–2215.

Gavrilets, S. 2004. *Fitness Landscapes and the Origin of Species*. Princeton University Press, Princeton.

Gavrilets, S. & Boake, C.R.B. 1998. On the evolution of premating isolation after a founder event. *Am. Nat.* **152**: 706–716.

Genner, M.J., Seehausen, O., Lunt, D.H., Joyce, D.A., Shaw, P.W., Carvalho, R. & Turner, G.T. 2007. Age of Cichlids: new dates for ancient lake fish radiations. *Mol. Biol. Evol.* **24**: 1269–1282.

Greenwood, P.H. 1989. The taxonomic status and phylogenetic relationships of Pseudocrenilabrus Fowler (Teleostei, Cichlidae). *Ichthyological Bulletins of the J.L.B. Smith Institute of Ichthyology* **54**: 1–16

Hendry, A.P., Wenburg, J.K., Bentzen, P., Volk, E.C. & Quinn, T.P. 2000. Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. *Science* **290**: 516–518.

Hendry, A.P., Taylor, E.B. & McPhail, J.D. 2002. Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the misty system. *Evolution* **56**: 1199–1216.

Hollander, J., Lindegarth, M. & Johannesson, K. 2005. Local adaptation but not geographical separation promotes assortative mating in a snail. *Anim. Behav.* **70**: 1209–1219.

Jackson, P.B.N. 1961. *The Fishes of Northern Rhodesia. A Check List of Indigenous Species*. Luska, Zambia.

Jiggins, C.D., Naisbit, R.E., Coe, R.L. & Mallet, J. 2001. Reproductive isolation caused by colour pattern mimicry. *Nature* **411**: 302–305.

Jiggins, C.D., Salazar, C., Linares, M. & Mavarez, J. 2008. Hybrid trait speciation and Heliconius butterflies. *Philos. Trans. R. Soc. B Biol. Sci.* **363**: 3047–3054.

Joyce, D.A., Lunt, D.H., Bills, R., Turner, G.F., Katongo, C., Duftner, N., Sturmbauer, C. & Seehausen, O. 2005. An extant cichlid fish radiation emerged in an extinct Pleistocene lake. *Nature* **435**: 90–95.

Kassam, D.D., Adams, D.C., Hori, M. & Yamaoka, K. 2003. Morphometric analysis on ecomorphologically equivalent cichlid species from Lakes Malawi and Tanganyika. *J. Zool.* **260**: 153–157.

Katongo, C., Koblmuller, S., Duftner, N., Makasa, L. & Sturmbauer, C. 2005. Phylogeography and speciation in the *Pseudocrenilabrus philander* species complex in Zambian Rivers. *Hydrobiologia* **542**: 221–233.

Kawata, M., Shoji, A., Kawamura, S. & Seehausen, O. 2007. A genetically explicit model of speciation by sensory drive within a continuous population in aquatic environments. *BMC Evol. Biol.* **7**: Article No. 99.

Kidd, M.R., Danley, P.D. & Kocher, T.D. 2006. A direct assay of female choice in cichlids: all the eggs in one basket. *J. Fish Biol.* **68**: 373–384.

Kilias, G., Alahiotis, S.N. & Pelecanos, M. 1980. A multifactorial genetic investigation of speciation theory using *Drosophila* m*elanogaster. Evolution* **34**: 730–737.

Koblmueller, S., Sefc, K.M., Duftner, N., Katongo, C., Tomljanovic, T. & Sturmbauer, C. 2008. A single mitochondrial haplotype and nuclear genetic differentiation in sympatric colour morphs of a riverine cichlid fish. *J. Evol. Biol.* **21**: 362–367.

Kodric-Brown, A. 1993. Female choice of multiple male criteria in guppies – interacting effects of dominance, coloration and courtship. *Behav. Ecol. Sociobiol.* **32**: 415–420.

Kondrashov, A.S. & Kondrashov, F.A. 1999. Interactions among quantitative traits in the course of sympatric speciation. *Nature* **400**: 351–354.

Leimar, O., Doebeli, M. & Dieckmann, U. 2008. Evolution of phenotypic clusters through competition and local adaptation along an environmental gradient. *Evolution* **62**: 807–822.

Maan, M.E., Hofker, K.D., van Alphen, J.J.M. & Seehausen, O. 2006. Sensory drive in cichlid speciation. *Am. Nat.* **167**: 947–954.

Martin, N.H. & Willis, J.H. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* **61**: 68–82.

Mayr, E. 1963. *Animal Species and Evolution*. Belknap, Cambridge.

McKinnon, J.S., Mori, S., Blackman, B.K., David, L., Kingsley, D.M., Jamieson, L., Chou, J. & Schluter, D. 2004. Evidence for ecology's role in speciation. *Nature* **429**: 294–298.

McPeek, M.A. & Wellborn, G.A. 1998. Genetic variation and reproductive isolation among phenotypically divergent amphipod populations. *Limnol. Oceanogr.* **43**: 1162–1169.

Mendelson, T.C. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (*Percidae: Etheostoma*). *Evolution* **57**: 317–327.

Mooers, A.O., Rundle, H.D. & Whitlock, M.C. 1999. The effects of selection and bottlenecks on male mating success in peripheral isolates. *Am. Nat.* **153**: 437–444.

Morris, M.R., Batra, P. & Ryan, M.J. 1992. Male-male competition and access to females in the Swordtail *Xiphophorus nigrensis. Copeia* **4**: 980–986.

Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nelson, C.M. 1995. Male size, spawning pit size and female mate choice in a lekking cichlid fish. *Anim. Behav.* **50**: 1587–1599.

Noor, M.A. 1995. Reinforcement in speciation. *Trends Ecol. Evol.* **10**: 492–492.

Noor, M.A.F. 1999. Reinforcement and other consequences of sympatry. *Heredity* **83**: 503–508.

Nosil, P. 2007. Divergent host plant adaptation and reproductive isolation between ecotypes of *Timema cristinae* walking sticks. *Am. Nat.* **169**: 151–162.

Nosil, P. & Crespi, B.J. 2006. Ecological divergence promotes the evolution of cryptic reproductive isolation. *Proc. R. Soc. B Biol. Sci.* **273**: 991–997.

Ostbye, K., Naesje, T.F., Bernatchez, L., Sandlund, O.T. & Hindar, K. 2005. Morphological divergence and origin of sympatric populations of European whitefish (Coregonus lavaretus L.) in Lake Femund, Norway. *J. Evol. Biol.* **18**: 683–702.

Plenderleith, M., van Oosterhout, C., Robinson, R.L. & Turner, G.F. 2005. Female preference for conspecific males based on olfactory cues in a Lake Malawi cichlid fish. *Biol. Lett.* **1**: 411–414.

Podos, J. 2001. Correlated evolution of morphology and vocal signal structure in Darwin's finches. *Nature* **409**: 185–188.

Ramsey, J., Bradshaw, H.D. & Schemske, D.W. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* **57**: 1520–1534.

Reynolds, R.G. & Fitzpatrick, B.M. 2007. Assortative mating in poison-dart frogs based on an ecologically important trait. *Evolution* **61**: 2253–2259.

Ruber, L. & Adams, D.C. 2001. Evolutionary convergence of body shape and trophic morphology in cichlids from Lake Tanganyika. *J. Evol. Biol.* **14**: 325–332.

Rundle, H.D. 2003. Divergent environments and population bottlenecks fail to generate premating isolation in *Drosophila pseudoobscura. Evolution* **57**: 2557–2565.

Rundle, H.D. & Nosil, P. 2005. Ecological speciation. *Ecol. Lett.* **8**: 336–352.

Rundle, H.D., Mooers, A.O. & Whitlock, M.C. 1998. Single founder-flush events and the evolution of reproductive isolation. *Evolution* **52**: 1850–1855.

Rundle, H.D., Nagel, L., Boughman, J.W. & Schluter, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287**: 306–308.

Schliewen, U., Rassmann, K., Markmann, M., Markert, J., Kocher, T. & Tautz, D. 2001. Genetic and ecological divergence of a monophyletic cichlid species pair under fully sympatric conditions in Lake Ejagham, Cameroon. *Mol. Ecol.* **10**: 1471–1488.

Schluter, D. 1996. Ecological speciation in postglacial fishes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **351**: 807–814.

Schluter, D. 2000. *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.

Schluter, D. 2001. Ecology and the origin of species. *Trends Ecol. Evol.* **16**: 372–380.

Schluter, D. & Price, T. 1993. Honesty, perception and population divergence in sexually selected traits. *Proc. R. Soc. Lond. B Biol. Sci.* **253**: 117–122.

Seehausen, O. 2006. African cichlid fish: a model system in adaptive radiation research. *Proc. R. Soc. B Biol. Sci.* **273**: 1987–1998.

Seehausen, O. & Van Alphen, J.J.M. 1998. The effect of male coloration on female mate choice in closely related Lake

Victoria cichlids (*Haplochromis nyererei* complex). *Behav. Ecol. Sociobiol.* **42**: 1–8.

Seehausen, O., Van Alphen, J.J.M. & Witte, F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* **277**: 1808–1811.

Seehausen, O., Terai, Y., Magalhaes, I.S., Carleton, K.L., Mrosso, H.D.J., Miyagi, R., van der Sluijs, I., Schneider, M.V., Maan, M.E., Tachida, H., Imai, H. & Okada, N. 2008. Speciation through sensory drive in cichlid fish. *Nature* **455**: U620–U623.

Servedio, M.R. 2001. Beyond reinforcement: the evolution of premating isolation by direct selection on preferences and postmating, prezygotic incompatibilities. *Evolution* **55**: 1909–1920.

Servedio, M.R. 2004. The evolution of premating isolation: local adaptation and natural and sexual selection against hybrids. *Evolution* **58**: 913–924.

Simpson, G.G. 1944. *Tempo and Mode in Evolution*. Columbia University Press, New York.

Skelton, P.H. 1991. Pseudocrenilabrus. In: *Check-List of Freshwater Fishes of Africa (CLOFFA)*, Vol. 4 (J. Daget, J.P. Gosse, G.G. Teugels & D.F.E.T. van den Audenaerde, eds), pp. 394–398. MRAC, Tervuren and ORSTOM, Paris, Brussels.

van der Sluijs, I., Van Dooren, T.J.M., Seehausen, O. & Van Alphen, J.J.M. 2008. A test of fitness consequences of hybridization in sibling species of Lake Victoria cichlid fish. *J. Evol. Biol.* **21**: 480–491.

Spitze, K. 1993. Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. *Genetics* **135**: 367–374.

Terai, Y., Seehausen, O., Sasaki, T., Takahashi, K., Mizoiri, S., Sugawara, T., Sato, T., Watanabe, M., Konijnendijk, N.,

Mrosso, H.D.J., Tachida, H., Imai, H., Shichida, Y. & Okada, N. 2006. Divergent selection on opsins drives incipient speciation in Lake Victoria cichlids. *PLoS Biol.* **4**: 2244–2251.

Turelli, M., Barton, N.H. & Coyne, J.A. 2001. Theory and speciation. *Trends Ecol. Evol.* **16**: 330–343.

Vines, T.H. & Schluter, D. 2006. Strong assortative mating between allopatric sticklebacks as a by-product of adaptation to different environments. *Proc. R. Soc. B Biol. Sci.* **273**: 911–916.

Supporting information

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

Figure S1 Principal component plots of 13 morphological characters indicating the area of morphospace occupied by the four populations tested in mate choice experiments.

Table S1 Mean and standard deviations of the 13 linear morphological measurements per group.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.