

CHAPTER NINE

Progressive levels of trait divergence along a ‘speciation transect’ in the Lake Victoria cichlid fish *Pundamilia*

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Introduction and outline

Identifying mechanisms of speciation has proven one of the most challenging problems in evolutionary biology, perhaps mainly for two reasons, speciation is not readily accessible to experimental approaches, and rarely to time series analyses. Any one case of speciation can usually be investigated only at a single stage of completion. Cases of parallel ecological speciation driven repeatedly within the same taxon by divergent selection along replicate environmental gradients, have therefore received considerable attention (Schluter & Nagel 1995; Rundle *et al.* 2000). Several such systems have become major model systems in evolutionary ecology research, including sticklebacks in postglacial lakes (Rundle *et al.* 2000), *Heliconius* butterflies (Mallet *et al.* 1998), leaf beetles (Funk 1998) and *Timema* walking sticks (Nosil *et al.* 2002). They provide powerful means of identifying causes of divergence and may lend themselves to examining associations between variation in the environments and variation in the progress towards speciation (Nosil & Harmon, this volume). However, variation in the progress towards speciation among disconnected populations undergoing parallel speciation may be due to different contingency as much as different environments (Taylor & McPhail 2000). Ideally, to trace the correlates of the transition from panmixis to incipient speciation, one would want to study variation in the progress towards speciation in exactly the same pair of species, and along a continuous progress series, to minimize the potential confounding effect of variable historical contingency.

The cichlid fish species *Pundamilia pundamilia*, *Pundamilia nyererei*, their hybrids and intermediate colour morphs occur at various stages of differentiation at different islands placed along an environmental gradient of water clarity in Lake Victoria. I will subsequently refer to this as a ‘speciation transect’. At the same time, populations occurring at different islands do exchange genes as evident from microsatellite-allele frequencies (Seehausen *et al.* 2008), and also from several observed cases of invasion of, and establishment in, previously unoccupied islands within the 15 years time window that I have collected community

data for. Hence, I propose that the *Pundamilia* 'speciation transect' is close to the ideal case where variation in the progress towards speciation can be studied in the same pair of species and along a continuous series. As such, it lends itself to the identification of the sequence of events in population divergence that lead to the emergence of non-random mating and built-up of genetic differentiation between non-allopatric incipient species. In this Chapter, I review our current knowledge on this system, and use it to test predictions of alternative theoretical models of non-allopatric speciation.

I start by describing the phenotypes and their distribution patterns, then describe geographical variation in genetic and phenotypic differentiation, introducing the 'speciation transect', and review knowledge on causes of gene flow restriction. This will lead me to the experimental identification of traits involved in mate choice, their inheritance, the form of selection on male nuptial colouration and the sources of selection on female mating preferences. I will use current knowledge on the sources of selection to test alternative models of speciation. Finally, I will show male-male aggression is based on the same traits that female choice is based on; is a frequency-dependent source of selection; and facilitates the coexistence of incipient species. I conclude with a short summary of the main points and perspectives for future research.

Distribution and phenotypic characterization of *P. pundamilia* and *P. nyererei*

The genus *Pundamilia* is endemic to Lake Victoria (Seehausen *et al.* 1998a), including the upper Victoria Nile (pers. obs.). The two best studied species are *P. pundamilia* and *P. nyererei*. Both are found exclusively over rocky substrates. *P. pundamilia* has been recorded from almost every sampled patch of rocky habitat along the mainland shores of the lake, and also from most offshore islands (Seehausen *et al.* 1998a). The geographical range of *P. nyererei* is nested within that of *P. pundamilia*. *P. nyererei* has only been found in places where *P. pundamilia* is present (Seehausen & van Alphen 1999), and it is absent from large stretches of mainland shore and islands despite apparently suitable habitat (Seehausen 1996; Fig. 9.1).

Both species are of small to medium size, of generalized haplochromine shape, but with somewhat specialized unicuspid and recurved teeth, and they are morphologically highly similar (Seehausen 1996; Magalhaes *et al.*, in press). Females of both species are cryptically yellowish, brownish or greyish depending on the population, with a number of distinct darker vertical bars on the flanks. The vertical bars in the much larger males are black. The flanks of male *P. pundamilia* are blue-grey between the bars, with a bright metallic blue spinuous dorsal fin and red soft dorsal, caudal and anal fins. Males of *P. nyererei* instead are bright yellow between the bars on the lower flanks and bright crimson red above the lateral line. Their entire dorsal fin is bright crimson, and the other

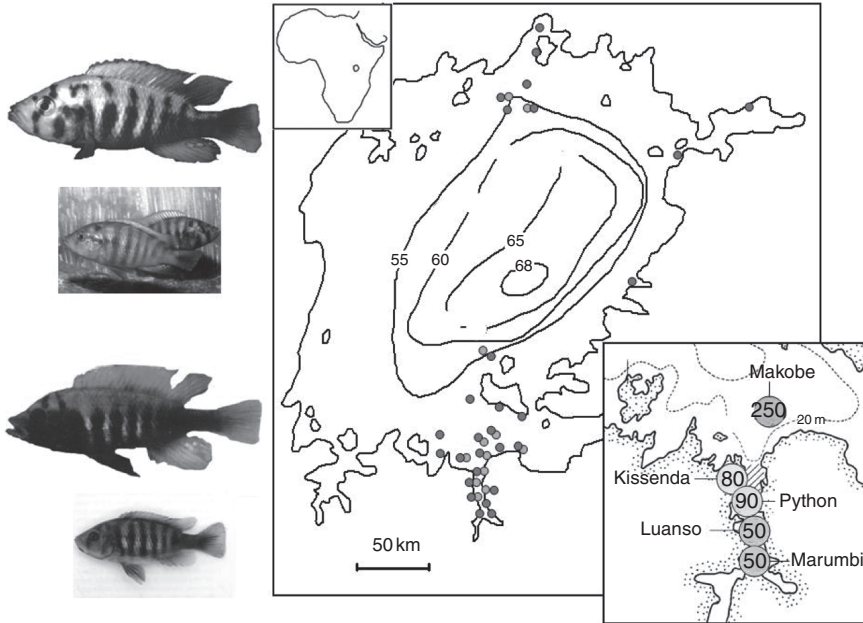


Figure 9.1 Male and female of *P. pundamilia* (top) and *P. nyererei* (bottom), both from Makobe Island (DS4). Large map: distribution of known records of *P. pundamilia* and *P. nyererei* in Lake Victoria. Small inset map: the ‘speciation transect’ with location of the five islands: Marumbi (DS1), Luanso (DS1), Python (DS3), Kissenda (DS2) and Makobe (DS4). Water transparencies at the islands (cm Secchi disk readings) are given inside the circles (colours: turbid = brown, clear = blue). Note that Kissenda Island lies inside a lateral embayment, hence the lower transparency than at Python Island despite Kissenda’s greater proximity to the clear water areas. (see colour plate)

unpaired fins are pale orange to red (Fig. 9.1). Adult males of both species are two to three times larger and heavier than adult females.

Geographical variation in the degree of genetic and phenotypic differentiation

I will review in this section population genetic, quantitative genetic and phenotypic evidence which suggest that, in different parts of their shared distribution range, fish of *P. pundamilia*- and *P. nyererei*-like phenotype are either well-differentiated sibling species, weakly differentiated incipient species, or merely extremes along a continuum of phenotypic variation.

Variation in male colour phenotypes can be described by a colour-based hybrid index (Fig. 9.2). The frequency distribution of hybrid index scores changes along a transect through increasingly clear waters, from unimodal in turbid water with predominantly intermediate phenotypes and complete absence of class 4 phenotypes to distinctly bimodal with predominantly

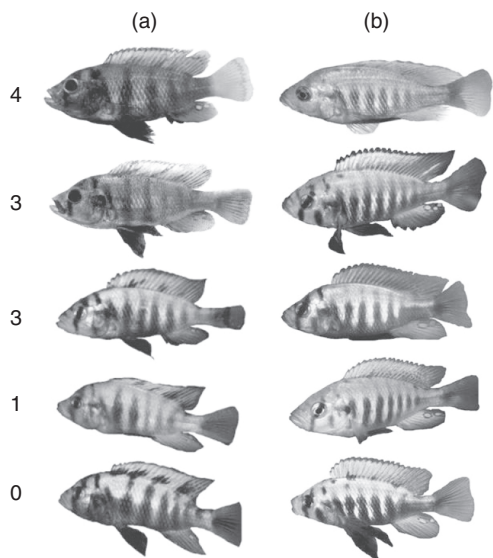


Figure 9.2 Colour-based hybrid index. Class 0 (*P. pundamilia*-like) = blue-grey lower flanks (lf), blue-grey upper flanks (uf), blue spinous dorsal fin. Class 1 = yellow lower flanks, grey upper flanks, blue spinous dorsal fin. Class 2 = yellow lf, red along upper lateral line, blue spinous dorsal fin. Class 3 = yellow lf, red uf except grey crest, blue dorsal fin. Class 4 (*P. nyererei*-like) = yellow lf, red uf, red df. (a) wild males from Luanso Island. (b) F2 hybrid males generated by crossing F1 hybrids between *P. nyererei* and *P. pundamilia* from Python Islands (1992 lines, DS3). (see colour plate)

phenotype classes 0 and 4 and very few individuals of intermediate colour in clear water (Seehausen 1997; Seehausen *et al.* 1997; Seehausen *et al.* 2008; Fig. 9.3a). Because the transition occurs along a fairly straight line from the highly turbid southern Mwanza Gulf to the relatively clear Speke Gulf, I refer to this transect as ‘speciation transect’. Other than productivity and associated water turbidity and ambient light, no other environmental variables are known to correlate with this transition.

To assess gene flow, we genotyped between 20 and 50 individuals each of the red and the blue phenotypes at each of the five islands along ‘speciation transect’ using 11 microsatellite loci (Seehausen *et al.* 2008). These data confirm the inferences made from phenotypic data. Significant differentiation is observed in the clear water sites and F_{ST} decays with increasing turbidity from 0.03 (Makobe Island) to 0.000 (Luanso and Marumbi Islands). At the high turbidity end of the transect (Luanso and Marumbi Islands), the differentiation was not significantly different from zero, but it was significantly different from zero at the islands Kissenda and Python with intermediate clarity. All 11 loci behaved similar, none showing any differentiation at Marumbi or Luanso, yet 7 of 11 showing significant differentiation at Makobe. Patterns of F_{ST} between island populations of the same species revealed significant isolation by distance in *P. pundamilia* but not in *P. nyererei* where F_{ST} were generally low and some not significant (Seehausen *et al.* 2008).

We generated F2 hybrids between *P. nyererei* and *P. pundamilia* in the laboratory (Haesler & Seehausen 2005) to acquire independent evidence that the variation around an intermediate hybrid index mode in turbid water populations was due to allelic segregation at those loci that are fixed for alleles with opposite sign in

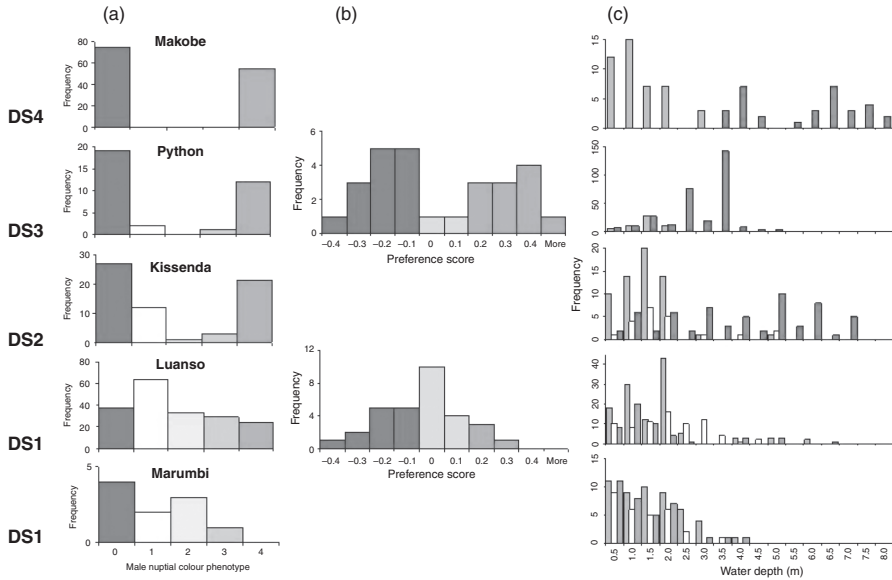


Figure 9.3(a) Frequency distributions of hybrid index scores along ‘speciation transect’. (b) Frequency distributions for female mating preference phenotypes at two stations along ‘speciation transect’. (c) Frequency distributions of males of different colour phenotypes against water depth. Males of *P. pundamilia*-like appearance (blue bars), *P. nyererei*-like appearance (red bars), reddish intermediates (hybrid index classes 2 and 3; orange bars), yellow intermediates (hybrid index class 1; pale yellow bars) DS1 to DS4 = Divergence score 1 to 4. (a) Modified from Seehausen *et al.* (2008). (b) and (c) reproduced with permission from Seehausen *et al.* (2008). (see colour plate)

the clear water areas. We used laboratory lines derived from populations of the two species collected in 1992 at Python Islands in the Mwanza Gulf (Seehausen 1996). By 1992, the frequency of phenotypic intermediates at Python Island was below 1% (Seehausen 1997). Our 1992 laboratory lines bred true. No intermediate male has appeared in the lines of either species and several hundred males have been bred and phenotyped in each line. More than 90% of the F1 hybrid males were intermediate in colour, resembling hybrid index class 3 (Fig. 9.2a), but some families contained a few blue males resembling class 0 or 1. The F2 hybrid males bred from both types of F1 male phenotypes (classes 0 and 3) segregated for colour. The phenotype classes in these F2 hybrid males resembled in great detail those in the wild males from turbid water areas (Fig. 9.2b). The close resemblance of wild phenotypes from turbid waters and laboratory hybrids between the species from clear water makes it likely that the same loci and alleles are responsible for the segregation of colour in both cases.

Based on the combined phenotypic and genetic evidence, I am henceforth assigning a divergence score (DS) to each island: DS1, Marumbi and Luanso; DS2, Kissenda; DS3, Python; and DS4, Makobe (Fig. 9.3).

Causes of gene flow restriction between incipient species

In the following I will show absence of intrinsic postzygotic isolation, and that divergent spawning site choice may restrict gene flow but strongly only at Makobe (DS4). In contrast, divergent female mating preferences are suitable to restrict gene flow at all stages of differentiation, but apparently only do so where a minimum water transparency is given.

No intrinsic postzygotic isolation

All Lake Victoria cichlid species that have been tested in the laboratory produce viable and fertile hybrid offspring (Crapon de Caprona & Fritzsche 1984; Seehausen *et al.* 1997). *P. pundamilia* and *P. nyererei* are fully interfertile too, and no intrinsic hybrid disadvantage could be detected in experiments in which a number of fitness-related variables were studied using Python Island fishes (DS3; Van der Sluijs *et al.* 2008b). Gene flow restrictions between the species in nature must hence arise from prezygotic or extrinsic postzygotic mechanisms.

Spawning habitat choice

Males of *Pundamilia* are highly territorial and spawning takes place within the territories of the males (Seehausen *et al.* 1998b; Maan *et al.* 2004). Both direct mate choice by females and microhabitat selection by territorial males can potentially restrict gene flow between the species. At the clear water Makobe Island (DS4), *P. nyererei* males breed deeper than *P. pundamilia* males and overlap is very limited (Seehausen 1997). However, at Python (DS3) and Kissenda Islands (DS2), where the species are phenotypically strongly, but genetically only weakly (but significantly), differentiated, the microdistributions largely overlap. *P. nyererei* breeds from very shallow to deep, whereas *P. pundamilia* breeds only in the uppermost 3 m (Seehausen 1997; Seehausen *et al.* 2008). Microhabitat selection by males can then only have a minor effect on restricting gene flow. Similarly, at Makobe Island, the spawning site distribution of *P. nyererei* is fully overlapping with that of a third *Pundamilia* species, the blue-black *P.* 'pink anal'. Microhabitat selection by males cannot account for the restriction of gene flow between these phenotypically divergent species either (Seehausen *et al.* 1998b).

Direct female mate choice

Direct female mating preferences have been measured for females from the very clear island (Makobe Island, DS4), the very turbid one (Luanso Island, DS1) and the one with intermediate turbidity (Python Island, DS3) along 'speciation transect'. Six females tested from the clear Makobe Island had behavioural preferences for conspecific males; four of them spawned, all with conspecific males (Seehausen 1997). A larger number of females were tested from Python Island, and most females actively preferred conspecific males; however, a few

had no preference. For Fig. 9.3b I calculated the frequency distribution of female mating preference phenotypes, combining data from several studies and 27 different females (Seehausen & van Alphen 1998; Haesler & Seehausen 2005; Seehausen *et al.* 2008). The distribution is strongly bimodal, with most females having significant preferences either for *P. nyererei* (exclusively *P. nyererei* females) or for *P. pundamilia* males (exclusively *P. pundamilia* females) and very few having no preferences. It is hence likely that divergent female mating preferences restrict gene flow between the incipient species at Python Islands (DS3). However, it also seems likely that divergent selection must be acting on female preferences because the strong bimodality in the trait variance despite the presence of hybrids seems unlikely to be maintained in the absence of divergent selection.

Two studies have investigated female mating preferences in the turbid water population of Luanso Island (DS1), where the male phenotype frequency distribution is unimodal and dominated by males of intermediate colouration (Seehausen *et al.* 2008). The first study was conducted in 1997, using 16 females collected in 1996 (Seehausen 1999). The second study was conducted in 2005–2006 using 30 females collected in 2003 and 2005 (Van der Sluijs *et al.* 2008). Both studies found significant between-female variation in mating preferences. In contrast to the population from Python Island (DS3), the frequency distribution of preference phenotypes was unimodal, with most females lacking preferences, several having a preference for blue males and fewer having a preference for red males. The two studies conducted with a 9-year interval between them and by different researchers yielded highly congruent results. The coincidence of the absence of bimodality in the frequency distributions of both female mating preference and male colouration in the turbid waters is consistent with the hypothesis that divergent female mating preferences maintain non-random mating between the incipient species at the islands with clearer waters.

The mate choice traits and their inheritance

Two very different manipulation experiments implicate divergent male nuptial colouration as a target of the divergent female mating preferences. Females of the Python Island populations (DS3) that preferred conspecific males under broad spectrum illumination failed to behaviourally discriminate between con- and heterospecific males under narrow spectrum orange light illumination (Seehausen & van Alphen 1998). Recently, Stelkens *et al.* (2008) used a different approach. In their experiment, second-generation hybrid males, segregating for nuptial colour, competed for matings with *P. pundamilia* and *P. nyererei* females from Python Island (DS3) in a partial partition mate choice design (Turner *et al.* 2001). Comparing mating success between the more blueish and the more reddish hybrid males, using molecular paternity tests, Stelkens *et al.* showed that when competing for females of *P. nyererei*, reddish hybrid males had

significantly higher mating success than blueish hybrid males, whereas a strong trend approaching significance ($p = 0.07$) in the opposite direction was found when males were competing for *P. pundamilia* females. Overall, hybrid males had significantly higher mating success with females of the species whose male nuptial colouration they resembled than with the females of the other species (Fisher's combined probability test, $p = 0.02$). Importantly, the hybrid males were not produced by backcrossing but by mating F1 hybrids with each other. The result would only be expected if females of both species when choosing mates use male nuptial colouration, but prefer different colours or a trait that tightly cosegregates with colour through linkage or pleiotropy.

Three investigations have addressed the mechanism of mate preference acquisition in *Pundamilia*. Haesler and Seehausen (2005), using a quantitative genetics approach, were able to demonstrate a heritable basis of the divergence between *P. pundamilia* and *P. nyererei* in their mating preferences; 20 F1 hybrid females expressed inconsistent mate choice decisions and 19 of these showed no significant bias to males of either species. In contrast, significant preferences for either blue or red male phenotypes segregated among 30 F2 hybrid females. Using the Castle–Wright biometric estimator (Castle 1921; Wright 1968; Lande 1981), between 1 and 5 genes were estimated to contribute to the variation in preference. Van der Sluijs *et al.* (2008) studied 21 additional F2 hybrid females and obtained closely corresponding results, again suggesting oligofactorial inheritance of the female mating preference. Both studies used the weakly differentiated populations of Python Islands, which differ mainly in male colouration and female preferences (DS3).

It appears that these inherited preferences can be modulated by imprinting on as yet unknown, but perhaps olfactorial traits. Verzijden and ten Cate (2007), using a cross-fostering design, found a significant effect of species of foster mother on female mating preference. The effect was strong enough to reverse a preference in *P. nyererei* for conspecific males into one for heterospecific males. For this experiment the well-divergent populations of Makobe Island (DS4) were used, which besides differing in male colour and female preference also differ in depth distribution, diet and parasites (see p. 166, 168, Fig. 9.3c).

As discussed above, the differences in male nuptial colouration between *P. pundamilia* and *P. nyererei* are heritable too. Heritability has been shown also for the intraspecific variation in the brightness of the red colouration between populations of *P. nyererei* (Seehausen *et al.* 1997). In a quantitative genetics approach using hybridization between the incipient species from Python Islands (DS3), the frequencies of different phenotype classes in the F2 suggested epistasis between one major gene with two alleles ('blue-grey flank' and 'yellow flank') and at least two quantitative trait loci for the presence and extension of red upwards from the lateral line, which act epistatically on the 'yellow flank' allele Seehausen (unpublished data).

Maan *et al.* (2006c) investigated a similar (blue/yellow) colour polymorphism in a population of the closely related species *Neochromis omnicaeruleus* from Lake Victoria. They also found evidence for heritability, but additionally evidence for ontogenetic switches from yellow to blue in many males, with some males lacking the yellow phase altogether, and a few staying yellow through most, perhaps all of their lives. Similar ontogenetic colour changes have not been observed in *Pundamilia*, but the findings in *Neochromis* raise the possibility that divergence between species or genotypes with yellow-red and blue male colouration may have come about through selection on heterochrony of ontogenetic colour change.

The form of selection on male nuptial colouration

The experimental and observational data discussed until here suggest that the maintenance of the phenotypic divergence between *P. pundamilia* and *P. nyererei* involves divergent or disruptive selection on male nuptial colouration within island populations. It is possible, and perhaps plausible, that the same applies to the origin of these divergent phenotypes. Geographical distribution patterns of species and of colours in *Pundamilia* suggest both geographical and non-geographical speciation may have occurred. Besides its major peak on close to zero range overlap, the frequency distribution of species' range overlap has a second minor peak on close to 100% overlap whereas intermediate categories of range overlap are less frequent (Seehausen & van Alphen 1999). Moreover, the distribution of male colour patterns is significantly overdispersed, species of similar colour co-occur less often, and species of different colour more often than expected by chance, consistent with the hypothesis that colour pattern diversity is under negative frequency-dependent selection (Seehausen & Schluter 2004).

Negative frequency-dependent selection on male colour could arise from male-male aggression (Seehausen & Schluter 2004). Dijkstra *et al.* (2007a), investigating effects of male colouration on male-male interactions, found that males of clear water populations (DS4) of *P. pundamilia*, *P. nyererei* and *P. 'pink anal'* (another closely related blue species) direct more aggression to conspecific than to heterospecific intruder males, and that these aggression preferences can be eliminated by masking of colour differences under narrow-spectrum light. Dijkstra *et al.* (2005) found that males with red colouration had a significant advantage over blue males in dyadic interactions between *P. nyererei* and *P. pundamilia* males from Python Island (DS3), and this effect too was at least partially eliminated by masking of colour differences under narrow-spectrum light. Finally, Dijkstra *et al.* (in press), studying experimental assemblages of red and blue males from Kissenda island, a population with very weak differentiation at neutral loci and relatively frequent phenotypic hybrids (DS2), found that red males received significantly less aggression in mixed assemblages than in assemblages consisting only of red males.

This is the most direct evidence of negative frequency-dependent selection on male colouration yet.

Laboratory experiments and field-based quantification of female mating preferences suggest that (1) intraspecific female mate choice within populations of *P. nyererei* exerts positive directional selection on the extension of the red area of males; male mating success in the clear waters of Makobe Island (DS4) correlates significantly with the extension of the red area on the body of males (Maan *et al.* 2004). In the more turbid waters at Kissenda Island (DS2) it correlates with the extension of red and yellow area (Maan 2006). (2) The strength of female mating preferences in *P. nyererei* (Maan 2006) and/or the evolutionary response in the male colouration (Seehausen *et al.* 1997) correlate positively with water clarity. (3) Female mate choice in the turbid water population of Luanso Island (DS1) can exert divergent or disruptive selection on male nuptial colouration; while most females lack any significant preference for red or blue male colouration and mate at random, some females prefer red males and some prefer blue males (van der Sluijs *et al.* 2008). Measuring mating success of F2 hybrid males with F2 hybrid females (both generated from Python populations, DS3) in a laboratory experiment, Stelkens *et al.* (2008) show that such intraspecific variation in female mating preferences can exert disruptive selection, whereby the more blue and the more red males obtain more matings than males of intermediate coloration. Hence, even though the random mating behaviour of F1 hybrid females will tend to cause bimodality in the male trait to decay (Bridle *et al.* 2006), the circumstance that distinct preferences for red and blue males segregate in higher-generation hybrid females will facilitate the build-up and/or maintenance of bimodality and positive associations between preference and trait genes.

The sources of selection on female mating preferences and testing predictions of speciation models

Complete allopatric speciation without gene flow upon secondary contact can be excluded for *P. pundamilia* and *P. nyererei* as should be apparent from the above. Independent of whether the current levels of gene flow are a primary or a secondary phenomenon (see discussion), the speciation transect allows us to identify the minimum set of traits that have to be under divergent selection to recruit assortative female mating preferences. Hence, I concentrate here on models of speciation with gene flow. Alternative theoretical models of speciation with gene flow often differ primarily in the sources of divergent selection on female mating preferences (Kirkpatrick & Ravigné 2002; but see Gavrillets 2004 for a different classification). I propose that data from the *Pundamilia* 'speciation transect' are suitable to test predictions from four distinct models: classical reinforcement, adaptive speciation, sensory drive speciation and speciation by parasite-mediated divergent sexual selection.

Classical reinforcement

Classical reinforcement of mating preferences can occur when recombination between divergent genomes produces gene combinations, which incur reduced intrinsic fitness to their bearer relative to the fitness of parental genotypes (Dobzhansky 1937, 1940; Muller 1939; Wilson 1965; Butlin 1987; Coyne & Orr 1997; Kirkpatrick & Ravigné 2002). Two studies have experimentally addressed components of intrinsic hybrid fitness in *Pundamilia*. Seehausen *et al.* (1997) found no evidence for reduced fertility through four generations of hybridization between *P. nyererei* and *Platytaeniodus degeni*. Van der Sluijs *et al.* (2008b) found no evidence for any reduction in survival, growth rate, fecundity or fertility, nor for skewed sex ratios in hybrids between *P. pundamilia* and *P. nyererei*. Given the geological youth of Lake Victoria (reviewed in Stager & Johnson 2008), and the very short speciation intervals required to account for its endemic species diversity (Seehausen 2006), intrinsic hybrid incompatibilities may not be expected. However, sex determining genes of variable strengths have been found segregating in other Lake Victoria cichlid fish, which can cause strongly skewed sex ratios (Seehausen *et al.* 1999), and perhaps the evolution of assortative mating (Lande *et al.* 2001). No such effects are apparent in interspecific hybrids within *Pundamilia*.

Our results do not rule out that Dobzhansky–Muller incompatibilities exist because if reduced fitness affects only some genotypes in F2 and higher hybrid generations, we might have failed to detect them. Molecular marker-aided studies are required to identify Muller–Dobzhansky incompatibilities through comparison of observed with expected frequencies of genotypes in hybrid families. However, if rare, such incompatibility mutations may become purged soon after hybridization started, and before selection would have effectively reinforced mating preferences. Our laboratory experiments do not rule out reinforcement through selection against ecological hybrid intermediacy (see next paragraph). To the extent that the relationship between the degree of habitat overlap and the extent of divergence in mating preferences can be taken as a test, the observed negative relationship (Fig. 9.3) is consistent with gene flow constraining mate preference evolution as opposed to the positive relationship predicted by reinforcement.

Adaptive speciation

In adaptive speciation driven by negative frequency-dependent selection on resource utilization exerted by competition (Rosenzweig 1978; chapters in Dieckmann *et al.* 2004), variation in mating preferences is recruited by selection to generate assortative mating within resource use types. In species with sexual selection and sexually dimorphic traits, this may happen when variation in a mate choice signal (e.g. nuptial colour) becomes a marker trait for variation in traits related to resource utilization (e.g. the shape of the feeding structures).

Assortative mating between females with a preference and males with the matching trait might then evolve through ecological reinforcement of mating preferences (Kondrashov & Kondrashov 1999; Kirkpatrick 2001). The species *P. pundamilia* and *P. nyererei* typically differ in several ecological traits when they live in clear water, but are ecologically very similar in turbid water. To test one prediction of the adaptive speciation model, Mrosso *et al.* (manuscript) studied dietary variation using the stable isotope ratios $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, phenotypic variation in male colouration, and variation at neutral genetic loci using 11 microsatellites. The ratio $^{13}\text{C}/^{12}\text{C}$ is a measure of the extent to which an individual fish fed from the benthic versus the limnetic food chain; the ratio $^{15}\text{N}/^{14}\text{N}$ is a measure of the trophic level that an individual fish fed at. This was done in a DS1, a DS3, and in a DS4 *Pundamilia* community along the 'speciation transect'. Adaptive speciation makes the prediction that dietary differentiation coincides with, or precedes, phenotypic and neutral genetic differentiation.

In turbid waters, where variation in male colour phenotypes was unimodal (DS1), all individuals fell into a single tight cluster in the two-dimensional isotope space, as well as in multilocus genotype space. At Python Island with intermediate water clarity (DS3), there was considerably more individual variation in both isotope ratios, yet the ranges of variation of reds and blues on both axes completely overlapped with no significant differentiation between them. Despite the lack of dietary differentiation, red and blue males had significantly different microsatellite allele frequencies, and intermediate colour phenotypes were rare. In clear water (DS4), red and blue males formed non-overlapping clusters along the carbon isotope axis, highly significantly different allele frequencies at microsatellites, and there were no intermediate colour phenotypes. In line with the differences in microdistribution (see above and below), blue males had less and red males more strongly negative $^{13}\text{C}/^{12}\text{C}$ ratios, indicating that blues fed more on the benthic food chain and reds more on the limnetic food chain. Their trophic levels ($^{15}\text{N}/^{14}\text{N}$) were not different. These data suggest assortative mating is sufficiently strong to permit species differentiation at neutral loci even in the absence of significant dietary differences. It seems hence unlikely that species differences in female mating preferences for male nuptial colour variants evolved or are maintained because colouration was a marker trait of feeding specialization at the incipient stage of speciation.

Sensory drive speciation

In sensory drive speciation, assortative mating emerges as a by-product of adaptive divergence in a sensory system that is used in mate choice (Endler 1992; Boughman 2001, 2002). We measured the microdistribution (water depth and distance off shore) of males with different colours at several islands along

'speciation transect'. Differentiation in microdistribution between red and blue coincided closely with divergence in colouration and in allele frequencies at neutral loci. At islands where phenotypic variation in male colour was unimodal and genetic differentiation absent (DS1), more reddish and more bluish males had identical microdistributions. At the islands with intermediate water clarity (DS2, 3) - where variation in male colour was bimodal and neutral genetic differentiation weak but significant - red males extended into much greater depth than blue males. Finally, at the clear water island (DS4), where allele frequencies at microsatellites and colour were well differentiated, red males were restricted to deep water and blue males to shallow water with little overlap (Fig. 9.3c; Seehausen *et al.* 2008, see also Seehausen 1997).

These data are consistent with the sensory drive model of speciation. The ambient light spectrum in Lake Victoria shifts towards red with increasing water depth (Seehausen *et al.* 1997). At sites where the *Pundamilia* species are significantly differentiated (DS2-4), the red males hence tend to be found in relatively more red-shifted light environments. Sensory drive makes the prediction that the species differ in their visual properties such that *P. nyererei* sees red light better than *P. pundamilia* and vice versa for blue light. Until very recently two studies had addressed this question. Carleton *et al.* (2005) determined the wavelength of maximum absorbance (λ_{\max}) of cones and rods by microspectrophotometry in seven populations of four closely related species of *Pundamilia*, varying in visual environment and male nuptial colour. They also sequenced the six by then known opsin genes of the same individuals (subsequently, a seventh opsin gene was discovered; Spady *et al.* 2003). Microspectrophotometry determined that the λ_{\max} of the rod pigment and of two of the three expressed cone pigments were similar in all five species and all populations. However, the long wavelength sensitive (LWS) cone pigment varied among species, with 3-4 nm shifts in λ_{\max} . The shift coincided with two amino acid substitutions in the part of the LWS opsin protein that is directed into its retinal binding pocket and predicted to affect absorbance properties. The subtle shifts in λ_{\max} coincided with large shifts in male body colour, with longer LWS pigments being confined to individuals of red species and shorter ones largely confined to individuals of blue species. Furthermore, there were indications that *P. nyererei* may have a higher proportion of red/red versus red/green double cones, which might bias its absorbance further towards red.

Maan *et al.* (2006) used the optomotor response paradigm to compare the sensitivity of *P. pundamilia* and *P. nyererei* (Makobe populations, DS4) to narrow spectrum light of blue and red colour. They found that *P. pundamilia* had a significantly lower detection threshold for blue light but a significantly higher detection threshold for red light than *P. nyererei*. Hence, as predicted by the sensory drive hypothesis, the species that has red nuptial colouration was more sensitive to red light, whereas the species with blue nuptial colouration was

more sensitive to blue light. A first comprehensive test of the sensory drive hypothesis has just been published. Comparing the divergence in alleles at opsin genes and nuptial colouration is currently at five sites along the 'speciation transect', Seehausen *et al.* (2008) found evidence consistent with speciation through sensory drive without geographical isolation.

Parasite-mediated divergent sexual selection

Good genes models of sexual selection are often thought of as constraining population divergence and speciation (Kirkpatrick & Nuismer 2004). However, in a heterogeneous environment, this need not be true because local adaptation may select for different good genes indicators, and hence different preferences in different parts of the environment (Edelaar *et al.* 2004; Reinhold 2004). The red dorsal body colouration of male *P. nyererei* is carotenoid-based, and correlational evidence indicated that it may be a signal of parasite resistance (Maan *et al.* 2004, 2006). Supportive evidence derives from a laboratory study involving experimental infection of males (Dijkstra *et al.* 2007b). Thus, carotenoids may mediate a trade-off between sexual signalling and immune defense, which would perhaps make male red colouration in *P. nyererei* an honest signal of individual quality.

Maan *et al.* (2008) investigated the relationship between male nuptial colouration and parasite load in *P. pundamilia* from Makobe Island (DS4) and found that the extent of iridescent blue on the dorsal fin is negatively correlated with parasite load too. They further found that parasite infestation rates differed quantitatively between the species, in a way that correlates well with species differences in diet and microhabitat. *P. pundamilia*, the shallow water species with the benthic carbon isotope signature, mostly carries the Nematode *Contracaecum*. *P. nyererei*, the deeper water species with the limnetic carbon signature, predominantly carries parasitic copepods. At Makobe Island, egrets and cormorants aggregate in large numbers on the shoreline, covering the rocks with guano. As a result, the abundance of infectious *Contracaecum* stages on the rocky bottom is likely to be very high in shallow waters and to decrease with depth. In contrast, parasitic copepod loads (*Ergasilus lamellifer* and *Lamproglana monodi*) were higher in *P. nyererei*. Due to its zooplanktivorous, limnetic feeding style at Makobe Island, *P. nyererei* may experience increased exposure to these free-living, pelagic copepods relative to the shallower-dwelling and more benthic-feeding *P. pundamilia*. It is hence conceivable that parasite-mediated divergent sexual selection strengthens reproductive isolation between *P. pundamilia* and *P. nyererei* at Makobe Island. However, whether resistance trade-offs exist remains to be tested. To answer the question whether parasite-mediated sexual selection is divergent in the initial stage of speciation or only latches on later when the species have already diverged more strongly ecologically, parasitisation patterns will have to be investigated at multiple sites along the 'speciation transect'.

Colour-based aggression biases in males help stabilize incipient speciation

Own-type aggression biases of males can importantly contribute to the stabilization of coexistence between ecologically similar species with well-developed male territoriality (Mikami *et al.* 2004; Seehausen & Schluter 2004). Theoretical modelling suggests that if the traits under selection by male–male competition and female mate choice are the same, negative frequency-dependent selection exerted by male–male competition could be very important also for the likelihood of sympatric speciation (Van Doorn *et al.* 2004, cf. *magic trait*, Gavrilets 2004). Dijkstra *et al.* 2006 tested predictions of this idea by investigating male aggression biases in the blue species *P. pundamilia* at different stages of speciation along the ‘speciation transect’. In the very turbid water site Luanso (DS1), where *Pundamilia* forms a single fully admixed population with a single blue-shifted mode in the phenotype frequency distribution, all males tended to be more aggressive towards blue males than to red males. This would favour the relatively rare more reddish phenotypes.

At sites with intermediate water clarity (Kissenda and Python Islands), two modes in the male phenotype frequency distribution, but persisting low levels of hybridization (DS2 and 3), both red and blue males biased aggression towards red males. This was not predicted by the hypothesis, which had predicted own-type aggression biases. However, red males are more abundant at these islands (Seehausen 1997). Moreover, the disadvantage that red males would experience due to aggression bias against them was offset by a significant behavioural dominance advantage of red over blue males in dyadic contests (Dijkstra *et al.* 2005). It is possible that the joined action of these forces does indeed stabilize coexistence at this intermediate stage of species divergence. Consistent with this, Dijkstra *et al.* (in press) found significantly lower levels of aggression in mixed than in entirely red experimental assemblages. They used males from Kissenda Island (DS2).

Finally, in two sites with clear water and well-differentiated species (DS4), males of both species biased their aggression to conspecifics as predicted by the theory. Hence, even though the distribution of patterns in male–male competition and emergence of own-type biases along the ‘speciation transect’ are complex, divergent nuptial colours consistently have effects on male–male interactions. In turn, the male–male interactions facilitate both the invasion of a new colour and the coexistence of existing colours.

Discussion and perspectives

In this chapter, I have developed the argument that the *Pundamilia* ‘speciation transect’ comes fairly close to the wishful case where variation in the progress towards speciation can be studied in the same pair of species and along a continuous series of progress levels. As such, the *Pundamilia* ‘speciation transect’

is well suited to identify the sequence of events that lead to the emergence of non-random mating and subsequent built-up of genetic differentiation between non-allopatric incipient species. We do not know at present to what extent the lack of differentiation in turbid waters is a primary feature of those populations or is due to recent breakdown of reproductive isolation (Seehausen *et al.* 1997; Taylor *et al.* 2006). It is, however, almost certainly a combination of both. Turbidity has increased dramatically in the past century, and this has certainly led to increased hybridization. On the other hand, historical data (Graham 1929) show that even before the recent anthropogenic eutrophication, Lake Victoria had large inshore areas of turbid waters, including the southern Mwanza Gulf. There is also evidence that the lake underwent several cycles of eutrophication and re-oligotrophication since the beginning of the Holocene (Schmidt 2003). Finally, turbid water conditions were probably also prevalent during the early stage of refilling of Lake Victoria through flooding of a productive grassland (Stager & Johnson 2008), and haplochromine populations with blue/red variation in male nuptial colouration, similar to DS1 on the *Pundamilia* ‘speciation transect’, are known from several rivers and small turbid lakes in Tanzania. Hence, rather than being deterred by the uncertainty about the extent to which the ‘speciation transect’ is a primary or a secondary phenomenon, it should be seen primarily as a rare opportunity to investigate the sequence of divergence/convergence in different traits relative to that in mating patterns. It allows us to identify the minimum set of traits that have to be under divergent selection to recruit and/or maintain non-random mating.

A number of conclusions can be drawn from the investigations carried out to date.

1. All evidence suggests that the southern end of ‘speciation transect’ is inhabited by a single species of variable colour: colour and preference phenotypes have a unimodal frequency distribution; there is no association between colour and microdistribution or diet and there is no neutral genetic differentiation between the phenotypes. All evidence suggests that the northern end of ‘speciation transect’ is inhabited by two different species: colour and preference distributions are strongly bimodal with few or no intermediates; colour is strongly associated with microdistribution and diet, such that red and blue males have almost non-overlapping depth ranges and non-overlapping isotope signatures of diet. Finally, they have significantly different allele frequencies at many neutral loci.
2. The mate choice traits that mediate assortative mating at the differentiated (north) end of the transect (female preferences and male nuptial colours) are already variable at the non-differentiated (south) end of the transect. *Pundamilia* is not unusual in this regard. There is a large number of cichlid populations in Lake Victoria that segregate for the different nuptial colours

and in fact at various levels of water transparency (Seehausen & van Alphen 1999; Maan *et al.* 2006; Terai *et al.* 2006).

3. Populations from two intermediate sites on the transect are intermediate in that they show strongly bimodal frequency distributions of the mate choice traits, male nuptial colour and female preference, and weakly but significantly different allele frequencies between red and blue males at neutral loci. These populations also show an intermediate level of association between colour and microdistribution; reds have a much wider depth range, while blues are restricted to shallow waters. On the other hand, these populations show no evidence for an association between colour and isotope signature of diet.
4. The data are compatible with models of speciation by sensory drive, where divergent light conditions at different water depths and divergent adaptations of the visual system affect mating preferences and the fitness of male nuptial colour morphs. Detailed analyses of the visual system of populations from all stages of differentiation on the transect have very recently confirmed this (Seehausen *et al.* 2008).
5. The same nuptial colour cues that females use for choosing mates are also used by males when defending territories against competitors. Evidence gathered from a number of different experiments are consistent with the hypothesis that colour-mediated competition between males contributes a negative frequency-dependent selection component that may stabilize the coexistence of the incipient species.
6. Parasitological data have yet to be collected from intermediate sites on the transect to ask whether sexual selection for parasite resistance in different parts of the environment may contribute to divergent selection on mating preferences and colours. Imprinting experiments ought to be conducted with fish from intermediately differentiated and non-differentiated populations to better understand the role of imprinting in the early stages of speciation.
7. The accumulation of divergence in a successively larger number of traits along the *Pundamilia* 'speciation transect' can be equated with increasing dimensionality of niche divergence (Nosil & Harmon, this volume). One potentially important observation then is that the dimensionality of niche divergence can grow rapidly with decreasing gene flow or vice versa, gene flow can rapidly decrease with increasing dimensionality of niche divergence. That divergent adaptation and gene flow can be reciprocally constraining has been shown on other incipient species systems too (Hendry *et al.* 2002; Nosil & Crespi 2004). It will be difficult to decide who is driving who. If the analogy of the different stages of differentiation along the 'speciation transect' with successive stages in speciation is valid, the observations in *Pundamilia* may imply that dimensionality of niche divergence and gene flow restriction co-evolve. A minimum dimensionality of niche

divergence is required for divergent selection to be sufficiently strong to initiate speciation (here the occupation of a sufficiently large range of water depth to be exposed to different light environments). However, as soon as gene flow becomes sufficiently reduced, divergence in other traits becomes possible, which may be under less strong divergent selection. In turn again, the increased dimensionality of niche divergence is likely to reduce gene flow further.

Similar gradients of species differentiation that lend themselves to investigations of 'speciation transects' might exist also in other taxa to the extent that non-geographical speciation occurs. The highest likelihood of discovering them is in sister species pairs with fully sympatric or nested geographical distributions that are sufficiently large to generate some population structure and to cover a range of different environments.

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