The role of hybridization in adaptive evolution

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Vorgelegt von
Rike Bahati Stelkens
aus Deutschland

Leiter der Arbeit:
Prof. Dr. Ole Seehausen
Aquatische Ökologie und Evolution
Zoologisches Institut

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Prof. Dr. Urs Feller

*Novelties come from previously
unseen association of old material.
To create is to recombine.*

Francois Jacob (1977)

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Chapter 1

Introduction

Historical review

The role of hybridization in adaptive evolution has been neglected, for several decades, in evolutionary biology research. Despite important early contributions (Lotsy 1916, 1931; Anderson 1949; Anderson and Stebbins 1954; Stebbins 1959; Lewontin and Birch 1966; Stebbins 1966; Templeton 1981), the potential role in adaptation and speciation of genetic exchange between species has only recently begun to be explored again more thoroughly.

The belated interest in hybridization is not surprising when put into historical context. Since Linnaeus' *Systema Naturae* (1735) started the era of taxonomy in the early 18th century, it has been one of the principal aims for scientists to systematically identify, order and categorize organisms into species. While this immensely improved our understanding of biodiversity, those forms that did not fit distinct species descriptions fell into intermediate (hybrid) categories and were for a long time considered a faux-pas of nature and discarded as evolutionary dead-ends. Before the modern theory of evolution was accepted in the scientific community and the general public, hybridization posed a problem to the prevailing belief that species remain unchanged since their creation as interspecific matings obviously provided a means of species modification. For more than ten thousand years, since the beginning of domestication of plants and animals, humans have made explicit use of interspecific genetic exchange and the resulting occurrence of intermediate and novel phenotypes. It seems ironic now, that it had been neglected for so long that the processes used in agricultural breeding programmes are in fact analogous to natural hybridization as discussed by Darwin (1868). Darwin clearly had recognized the importance of hybrids as he writes:

> *Those forms which possess in some considerable degree the character of species, but which are so closely similar to some other forms, or are so closely linked to them by intermediate gradations, that naturalists do not like to rank them as distinct species, are in several respects the most important to us.* (from the chapter '*Hybridism*' (Darwin 1859)).

Also Linnaeus, despite his fundamentally creationists views, surprisingly acknowledged that species could arise through hybridization (Linnaeus (1760) as cited by Grant (1981), p 245). However, the introduction of the biological species concept (BSC) in the 20th century by Mayr and Dobzhansky, defining species as *groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups* (Mayr 1963), reinforced the efforts to investigate evolutionary mechanisms that keep species apart rather than those that lead to genetic exchange between them. Some argue that the eugenic political and societal views of the 19th and early 20th century contributed to the aversion, especially of zoologists, to deal with the genetic admixture of species (Mallet 2007). The natural occurrence of hybrid individuals was a nuisance to the practicality of the BSC and the fact that hybrids are often less viable and fertile than the parental species, was taken as a confirmation that hybridization is a 'breakdown of isolating mechanisms' (Mayr 1963), and merely an obstacle to further divergence of the "pure" lines. As a consequence, hybridization was considered to be an exceptional rather than a common event, and believed to almost never result in gene flow between species and introgression.

Despite the apathy, some scientists, amongst them mostly botanists, recognized and demonstrated the importance of hybridization for adaptation to new environments (Anderson and Stebbins 1954; Stebbins 1959) using cross-breeding experiments (Anderson and Hubricht

1938) and field studies to assess hybridization frequencies (Anderson 1948). They were later joined by zoologists who suggested that introgressed genes from other species may provide a source of genetic variation opening the way to novel evolutionary trajectories (Lewontin and Birch 1966). Since the advent of molecular genetics in the 1980s, evidence for natural hybridization kept accumulating from many different taxonomic fields and could no longer be ignored.

Hybrid zone models
This accumulation of evidence spawned a concerted effort to study hybrid fitness and the conditions under which hybrid populations may become established in the wild. Thanks to a series of theoretical studies from the 1970s to the present day, predictions can now be made on the dynamics of hybrid zones in geographical space. Models of hybrid zones can be classified as derivatives of two conceptually different models with respect to the fitness of hybrids relative to their parents (reviewed in Moore 1977; Harrison 1990). The first model assumes hybrid fitness to be generally lower than that of the parents independent of the environment. Although hybrids may be constantly produced in the contact zone between parental species, low intrinsic fitness due to endogenous selection against hybrids keeps the zone narrow (*tension zone model*) (Bazykin 1969; Barton and Hewitt 1985). In many models the width of the zone depends on the balance between dispersal abilities of the parental species into the range of the other species, and selection against hybrids (e.g. Haldane 1948; Fisher 1950). The other model argues, that hybrid zones are often maintained by exogenous selection favouring parental and hybrid genotypes in different environments (*bounded hybrid superiority model* (Moore 1977)) rather than by the balance between parental dispersal into the zone and endogenous selection against hybrids.

The shape of hybrid zones depends on the fitness of hybrid genotypes relative to non-hybrid genotypes making the following predictions: i) If both parents have an environment-dependent selective advantage over hybrids, the zone will stay narrow. ii) If parental and hybrid genotypes do not differ in fitness, the zone will widen and eventually result in the merging of the two parental gene pools. iii) If one parent is more fit than both the other parent and the hybrid, the zone will move through space like a 'traveling wave' (e.g. Blum 2002; Kawaguchi and Sasaki 2006). iv) If hybrids have an environment-dependent fitness advantage over both parents, the zone will be shaped by the distribution of habitat parameters. If the parental ranges coincide with an environmental cline, hybrid zones will follow the distribution of the ecotone (e.g. MacCallum et al. 1998). As habitat transitions are rarely smooth, this often results in mosaic-shaped hybrid zones (e.g. Vines et al. 2003).

The main conclusion of the different hybrid zone models is that the evolutionary fitness of hybrid genotypes is determined by the sum of endogenous and exogenous selection where intrinsic fitness is expected to be a function of genetic distance between species (endogenous selection) and extrinsic fitness is a function of ecological competitiveness with parental genotypes, i.e. availability of underutilized niche space (exogenous selection).

Hybridization as an evolutionary stimulus
In the past two decades, the understanding of the role of hybridization in evolution has experienced a profound paradigm shift, largely driven by discoveries due to the advent of molecular genetics. The availability of nuclear and mitochondrial markers has made gene flow between species and its consequences a main focus of evolutionary research (Dowling and Secor 1997). Using multilocus techniques and genome scans we can now identify hybrid individuals (Rieseberg and Linder 1999; Anderson and Thompson 2002) and lineages (Ungerer et al. 1998) more easily and track the threads of reticulate evolution with more confidence (Marri et al. 2007). It is now clear that hybridization between species is much more common than was thought previously. Two recent reviews on hybridization frequency

reveal that hybridization is the rule rather than the exception. At least 25% of all plant species (Mallet 2005) and on average 1% (Schwenk et al. 2008) to 10% (Mallet 2005) of animal species seem to regularly hybridize. Hybridization is particularly common in young and rapidly radiating species groups (Seehausen 2004; Mallet 2005). Equipped with the new molecular tools, we can now investigate the adaptive potential of interspecific hybridization. Although this research field is still in its infancy, we already have evidence covering a large diversity of taxa that hybridization can be a stimulus for adaptive evolution and phenotypic diversification (Grant and Grant 1992; Rieseberg et al. 2003a; Seehausen 2004; Arnold 2006; Mallet 2007; Schwenk et al. 2008).

Of course, gene flow can also lead to the coalescence of species into a hybrid swarm if effects of gene flow exceed those of selection (e.g. Ellstrand 1992; Rhymer and Simberloff 1996; Taylor et al. 2006; Seehausen et al. 2007) and even a low frequency of hybridization may slow down or prevent the differentiation of species across much of the genome (Wu 2001). These aspects of hybridization, which one might classify as destructive with regard to species diversity and adaptation, are certainly common and become increasingly more frequent today due to anthropogenic habitat alterations and effects of globalization leading, for example, to a reduction of environmental heterogeneity, removal of dispersal barriers, habitat loss, species translocations or the simplification of signalling pathways (Seehausen et al. 1997; Hendry et al. 2006; Seehausen 2006). However, because this thesis focuses on the diversity-enhancing aspects of interspecific hybridization this introduction provides an overview of the various mechanisms by which hybridization catalyses rather than retarding diversification.

Interspecific hybridization can enhance the additive genetic variation of a population (Lewontin and Birch 1966; Svärdson 1970). The total effect of introgression measured on continuous traits (here beak and body dimensions of Darwin's finches) has been estimated to be two to three orders of magnitude larger than the effect of mutation (Grant and Grant 1994). Further, a variety of experiments has shown that lateral gene transfer in prokaryotes (Ochman et al. 2000; Gogarten et al. 2002; Boucher et al. 2003; Marri et al. 2007; Vernikos et al. 2007) and introgression in plants (e.g. Arnold and Bennett 1990; Klier et al. 1991; Kim and Rieseberg 1999)) and animals (e.g. Parsons et al. 1993; Grant and Grant 2008) can lead to the acquisition of specific adaptive traits. To the extent that hybridization thus allows for a faster response to selection than if the population was genetically isolated, it could provide populations with the means to better cope with environmental changes (reviewed in (Arnold 2006). This has resulted for instance in geographic range expansion of introgressed taxa and niche shifts (Feder et al. 2003; Lexer et al. 2003b; Kolbe et al. 2004; Nolte et al. 2005).

Apart from the effects of increased heritability due to overall increased genetic variation and the transfer of functional gene complexes, hybridization can also lead to evolutionary novelty, i.e. to the *de novo* origin of adaptations and new species. Some suggest, that hybrid speciation can result from allopolyploidy, where hybrids combine the full chromosomal complements of both their progenitors (Stebbins 1947; Grant 1981; Arnold 1997; Rieseberg 1997; Chapman and Burke 2007). However, there is also increasing evidence for the occurrence of homoploid hybrid speciation, i.e. hybrid species that retain the base chromosome number of their parents. Cases of hybrid speciation have been conclusively demonstrated in animals (Dowling and De Marais 1993; Schliewen and Klee 2004; Schwarz et al. 2005; Gompert et al. 2006; Mavarez et al. 2006; Meyer et al. 2006) and plants (Arnold et al. 1990; Ferguson and Sang 2001; Schwarzbach and Rieseberg 2002; Lexer et al. 2003a). The idea has also received support from modelling studies (Ungerer et al. 1998; Buerkle et al. 2000; Buerkle and Rieseberg 2008) predicting that homoploid hybrid speciation is facilitated by ecological isolation. This is because hybrids that invade novel or extreme habitats can more easily escape from homogenizing gene flow and competition with the parental species (McCarthy et al. 1995; Dowling and Secor 1997). Recent genomic studies corroborate the

view that hybridization can induce major ecological transitions by demonstrating that hybridization can provide the genetic variation necessary for entering into new discrete niches, a step that is difficult to make through mutation alone because it usually requires simultaneous changes of multiple traits (loci) (Rieseberg et al. 2003a; Gompert et al. 2006; Rogers and Bernatchez 2007).

In summary, research in the last 20 years has demonstrated that hybridization can have effects like a multi-locus 'macro-mutation' reaching out over large phenotypic distances (Barton 2001) and facilitate the colonization of underutilized peaks on adaptive landscapes (Mallet 2007).

Hybridization in adaptive radiations

Recently, the debate about the potential for hybridization to bring about genetic variation, functional novelty and new species reached a new level. Although Stebbins (Stebbins 1959), Lewontin and Birch (Lewontin and Birch 1966) and Templeton (Templeton 1981) developed similar concepts, only a few years ago Seehausen (2004) fully verbalized a potential explanation for the elevated variability and increased rates of phenotypic evolution observed in adaptive radiations.

Seehausen reviewed published molecular phylogenies and summarized the rather strong evidence for reticulate evolution found in many radiations, amongst them Darwin's finches (Freeland and Boag 1999), Hawaiian picture-winged fruit flies (DeSalle and Giddings 1986), Hawaiian crickets (Shaw 2002), Hawaiian silverswords (Baldwin et al. 1991; Barrier et al. 1999), African cichlid fish (Salzburger et al. 2002; Smith et al. 2003) and Lake Baikal sculpins (Hunt 1997; Kontula et al. 2003). Strikingly, all of these radiations have in common that the evolutionary history proposed by mitochondrial genes (monophyly) is discordant with the genealogies revealed by nuclear genes (polyphyly) suggesting that hybridization between genetically divergent species may be an important evolutionary mechanism triggering the rapid ecology-driven multiplication of lineages into new species.

This inspired the development of the *hybrid swarm origin hypothesis*, which argues that interspecific hybridization upon colonization of novel habitat can facilitate adaptive radiation (Seehausen 2004) and that the most rapid radiations are likely to be derived from two or more hybridizing ancestral taxa. It predicts that populations are more likely to undergo adaptive diversification rapidly if they become genetically 'enriched' by the admixture of divergent genomes. The latter can bring about large genetic variation at quantitative trait loci and new functional gene combinations that enable ecological specialization and niche segregation of genotypes with alternative specializations. The idea is further supported by the observation that the conditions causing contact zones between species and initiate hybridization often coincide with ecological opportunity conducive to adaptive radiation. Hybridization often takes place when habitats have been thoroughly altered, perturbed or newly colonized (e.g. during the drop or rise of lake water levels) (Templeton 1981). These habitat conditions can result in an increased accessibility of new or under-utilized fitness peaks (vacant niches) in an adaptive landscape that provide opportunity for ecological speciation associated with divergence along multiple different evolutionary trajectories.

During adaptive radiation into two or several ecologically differentiated species, the amount of standing genetic variation is likely to become eroded by strong divergent selection, as the populations track alternative ecological opportunities, which is only counteracted by mutation and immigration (Grant and Grant 2002). Seehausen (2004) proposes the *syngameon hypothesis*, which offers an explanation for why radiations may continue spawning new species even after the initial burst of speciation would have exhausted quantitative genetic variation. The *syngameon hypothesis* predicts that if members of a radiation occasionally or locally hybridize, new adaptive trait combinations can keep evolving at a higher rate than by mutation and immigration alone, which could prolong and maintain the momentum of

adaptive radiations (Gilbert 2003). Syngameon-like conditions have been observed in a variety of young radiations (e.g. Dowling and De Marais 1993; Grant and Grant 1996; Beltran et al. 2002). Following both the *hybrid swarm* and the *syngameon hypothesis*, the volume (species numbers and functional diversity) of a given adaptive radiation depends mainly on three factors i) the genetic distance between, and genetic variation within the founding lineages, ii) the opportunity for ecological speciation and iii) the opportunity for occasional hybridization between diverging populations within the radiation.

The *hybrid swarm theory* also provides some help to reconcile the opponents in the debate about sympatric speciation. Sympatric and parapatric speciation have long been thought (and still are by some) to be highly unlikely because establishing links between ecological genes and mating genes would require unrealistically strong disruptive selection to overcome the homogenizing effects of gene flow. However, theoretical models suggest that such speciation becomes more likely if the populations under disruptive selection possess large additive genetic variance on polymorphic genes for functional traits including phenotypic marker traits that can be recruited for mate choice (Higashi et al. 1999; Kondrashov and Kondrashov 1999). Interspecific hybridization provides populations with elevated levels of heritable genetic variation instantly. Hence, the chance for multiple functional genotypes to emerge simultaneously in sympatry is predicted to be higher after genetic admixture than in an isolated scenario with a narrower range of ecological trait values.

Hybridization is now thought to have played a role in the generation of adaptive radiations of prokaryotes (Vernikos et al. 2007), various groups of plants (Barrier et al. 1999; Rieseberg et al. 2003a), and animals (Feder et al. 2003; Seehausen 2004; Joyce et al. 2005), amongst them primates (Arnold 2006; Patterson et al. 2006) (reviewed in Seehausen 2004; Mallet 2007). There is even evidence that hybridization took place at the very beginning of life on Earth as the ancestral eukaryotic nuclear genome contains genes from multiple sources and may have resulted from a fusion of two or more diverse prokaryotic genomes (Doolittle 1999; Rivera and Lake 2004).

Hybridization in cichlid fish

The cichlid fish radiations in the Great African Lakes constitute the largest and most rapid adaptive radiations of vertebrates and are in the process of becoming one of the best model systems for studying both, adaptive radiation and the role of hybridization in adaptive evolution. In particular, the massive radiation of cichlid fish in Lake Victoria into >500 species and the short time window that geologists attribute to it has led to an interesting controversy about the origin of its genetic and phenotypic diversity (Johnson et al. 1996; Nagl et al. 2000; Seehausen 2002; Verheyen et al. 2003; Stager and Johnson 2007). Based on mitochondrial genealogies, the species flocks of Lakes Victoria and Malawi have, until recently, been thought to represent text book examples for monophyletic adaptive radiations until the first multilocus-nuclear phylogenies were published, suggesting otherwise (Seehausen et al. 2003). Nuclear genes revealed a poly- or paraphyletic origin, and the discordance with the mitochondrial genealogies suggested that several, genetically divergent species may have hybridized upon colonization of the lake basins. The different signature from mtDNA suggesting a single founding lineage can be explained by the fixation of one parental mtDNA haplotype during the course of subsequent radiation, a scenario finding empirical support from other fish species (DeMarais et al. 1992; Bernatchez et al. 1995) and many other animals (reviewed in Currat et al. 2008) and theoretical support in models simulating secondary contact with hybridization during range expansion (Excoffier and Ray 2008).

By now, several other large radiations of cichlid fish are suggested to have evolved after hybridization between two or more distantly related lineages (the radiation of Lake paleo-Makgadikgadi (Joyce et al. 2005), and the small and large tooth *Serranochromis,*

Sargochromis and *Pseudocrenilabrus* sub-radiations of Lake Mweru (data presented in Chapter 6 in this thesis)). Together, these results suggest that the admixture of divergent genomes before or at the onset of adaptive radiation is common in cichlid species radiations.

Apart from evidence for ancient hybridization at the base of adaptive radiations, there is in cichlid fish radiations also evidence for the hybrid origin of individual species within radiations (Schliewen and Klee 2004; Kidd et al. 2006; Koblmueller et al. 2007) and populations (Smith et al. 2003) of cichlid fish. Another line of research uses cichlids as a model system to investigate the effects of interspecific hybridization on the production of novel features and functions using laboratory crossing experiments (Albertson and Kocher 2001) and quantitative trait mapping, to identify regions of the cichlid genome responsible for functionally important shape differences between species, and to assess the underlying genetic architecture of novel phenotype expression (Albertson et al. 2003; Albertson and Kocher 2005; Parnell et al. 2008). A study using quantitative trait locus (QTL) sign tests investigated the selection history of specific morphological traits, and showed that genes underlying traits that experienced stabilizing or fluctuating selection, are more likely to produce phenotypic novelty in interspecific hybrids, than traits under directional selection (Albertson et al. 2003).

Intrinsic factors affecting the evolutionary potential of hybrids

Clearly, there are many obstacles on the way to hybrid speciation, the most apparent being that hybrids often suffer from fitness disadvantages or sterility due to genic incompatibilities. Most intrinsic hybrid inviabilities are believed to stem from Dobzhanzky-Muller-incompatibilities that can affect stage variable survival rates (e.g. fertilization success, hatching/emergence rates) (Dobzhansky 1936; Muller 1942; Lynch 1991; Edmands 1999; Gharrett et al. 1999; Turelli and Orr 2000). Incompatibilities accumulate as pleiotropic by-products of genetic drift or natural selection after populations become isolated and the genes causing incompatibilities are neutral or beneficial in their population of origin, but show negative epistasis when combined in a hybrid genome (Turelli and Orr 2000).

Because epistatic effects accumulate in a quadratic fashion in the Dobzhanzky-Muller model, incompatibility is predicted to first increase slowly with genetic distance, but later with accelerating speed. Intrinsic hybrid fitness disadvantages can also result from behavioural incompatibilities between males and females affecting the likelihood of heterospecific matings (e.g. male courtship location, time and behaviour, and sexual selection through female preference for male traits). The landmark studies by Coyne and Orr (Coyne and Orr 1989, 1997) introduced the now common approach of comparing the degree of hybrid premating isolation or postmating incompatibility to interspecific genetic distance and many laboratory hybridization experiments have since demonstrated that compatibility is strongly negatively correlated with genetic distance, and therefore with time since speciation (Edmands 2002; Price and Bouvier 2002; Bolnick and Near 2005).

Both experimental (Moll et al. 1965; Waser 1993; Marshall and Spalton 2000; Waser et al. 2000; Neff 2004; Willi and Van Buskirk 2005) and theoretical work (Campbell and Waser 1987; Schierup and Christiansen 1996) predict that the relationship between hybrid fitness and the genetic distance of parents is predicted to be hump-shaped with an 'optimal outcrossing distance' (Price and Waser 1979; Schierup and Christiansen 1996). Hybrids between very closely related parents (intraspecific, within-population crosses) may suffer from low fitness due to inbreeding depression, i.e. the expression of recessive deleterious mutations in homozygotes (Charlesworth and Charlesworth 1987). Then, as genetic distance increases (intraspecific, between-population crosses), fitness is predicted to rise due to heterosis, i.e. due to the beneficial effects of dominance and overdominance in heterozygotes. At larger genetic distances (distantly related intraspecific and interspecific crosses), hybrid fitness would decrease again due to outbreeding depression, i.e. due to the deleterious effects of genetic incompatibilities, the break-up of co-adapted gene complexes, epistatic interactions

and underdominance (heterozygote disadvantage) (Lynch 1991; Edmands 2002). Hence, the fitness peak will typically reside in the area of intraspecific between-population crosses. However, in rapidly radiating species flocks with many closely related species, the intrinsically determined fitness peak (i.e. excluding ecological fitness) may well be shifted into the interspecific area.

Following from this, hybridization is predicted to have the largest evolutionary effect after some morphological, ecological and genetic differences between species have arisen, but before the point is reached when genetic incompatibilities incur a severe fitness cost (Grant et al. 2004; Grant and Grant 2008). The capacity to produce viable hybrids between the ancestors of a putative hybrid species is therefore a key element when testing for its hybrid origin.

Extrinsic factors affecting the evolutionary potential of hybrids

Even in the case that hybrids carry favourable gene combinations, these must rise to high enough frequency for epistatic selection to be effective which can only be achieved by selection on whole hybrid populations (Barton 2001). In order to be stabilised and persist through time, it is therefore crucial that hybrid genomes become reproductively isolated from the parents. This constraint to hybrid speciation is particularly hard to overcome for homoploid hybrids that, unlike allopolyploid hybrids, do not automatically gain a post-zygotic mating barrier against the parents during meiosis. Under such conditions, the success of a hybrid critically depends on its ecological competitiveness with the parents. Theoretical (Buerkle et al. 2000) as well as experimental work (Abbott 1992; Jackson and Tinsley 2003; Lexer et al. 2003a) shows that hybrids are only likely to last if they can occupy an ecological niche that is sufficiently divergent from that of the parents. Yet, in most cases hybrids resemble one of the parents or express intermediate trait values lying between the parental means. Hence hybrids usually fall into a fitness valley between two adaptive peaks on an adaptive landscape. Without spatial isolation from the parents (which is the most common geographic scenario (Rieseberg and Willis 2007)) most hybrids are therefore not likely to persist (Barton and Hewitt 1985). The situation changes, if an underutilized fitness peak requires intermediate trait values (Mallet 2007), or if hybrids express phenotypes that are extreme or 'transgressive' compared to the parental species (as described above).

Transgressive segregation

Besides intermediacy, hybrids frequently express trait values that lie outside of the combined range of trait values of their parental species, a phenomenon referred to as transgressive segregation (Slatkin and Lande 1994; Rieseberg et al. 1999). Rieseberg and others reviewed data from 1229 traits in 171 experiments on animal and plant hybrids and found that 91% of studies reported at least one transgressive trait and that 44% of traits were transgressive overall. Transgressive segregation (TS) is commonly believed to result from complementary gene action, which describes the effect of alleles fixed for opposing signs on quantitative trait loci in the parents, that, when recombined in hybrid genotypes, can add up to an extreme (larger or smaller) overall trait value compared to the parents. Also other mechanisms have been suggested to cause TS in hybrids, such as non-additive effects by overdominance (where heterozygotes express more extreme trait value than both homozygotes at a particular locus) and epistasis (where the expression of one gene is modified by other genes) but quantitative genetic studies consistently identified complementary gene action as the primary cause of TS (Weller et al. 1988; De Vicente and Tanksley 1993; Mansur et al. 1993; Clarke et al. 1995; Ecke et al. 1995; Li et al. 1995; Monforte et al. 1997; Bradshaw et al. 1998; Xu et al. 1998; Kim and Rieseberg 1999; Rieseberg et al. 2003b).

TS can affect all kinds of quantitative traits, from morphological to physiological, but also behavioural and life history characters. Theoretically, TS can endow the phenotypic

diversification of a group with new momentum, because it instantaneously increases the working surface for selection, making it an interesting mechanism when investigating the ability of some hybrid lineages to invade ecological niches that neither parental species can occupy. Evidence, that the expression of extreme trait values in hybrids can increase the evolutionary potential of hybrids, comes from extensive studies on *Helianthus* sunflowers. A series of experiments over the last 20 years conclusively demonstrated how transgression in key ecological traits can pave the way to new ecological adaptations and speciation (e.g. (Schwarzbach et al. 2001; Welch and Rieseberg 2002; Lexer et al. 2003b; Rieseberg et al. 2003a; Gross et al. 2004). Also experiments on other plants (Zhang et al. 2001; Johnston 2004) and animals (Jackson and Tinsley 2003; Ranganath and Aruna 2003; Nolte et al. 2005) showed that hybrids can express trait values that enable them to tolerate ecological conditions beyond the initial ranges of both parental species, and hence enlarge a population's responsiveness to diversifying selection.

Thesis Outline and Summary of Chapters

Chapter 2 and 3 –Phenotypic novelty in interspecific hybrids is predicted by the genetic distance between the parental species

It is largely unknown how interspecific hybridization affects the rate and volume of functional diversification. One challenge is to quantify the amount and variety of adaptive novelty produced by hybridization. In **Chapter 2** and **3**, I investigated the previously untested prediction that the frequency of transgressive segregation in interspecific hybrids is a function of the genetic distance between the parental species (Rieseberg et al. 1999). The hypothesis is based on the expectation that with increasing time since speciation, the number of loci at which the parental species are fixed for alleles with opposite signs should increase, hence providing more frequent opportunities for complementary gene action between species with a longer history of independent evolution. When testing predictors of transgression, one must consider that the amount of transgression in hybrids can also depend on the magnitude of phenotypic divergence of the crossed species pair (Rieseberg et al. 1999). Phenotypically dissimilar species are less likely to produce transgressive offspring than more similar pairs. This is because consistent directional selection (driving phenotypic evolution into different directions) will more likely cause the fixation of alleles with opposite signs, eventually leading to the elimination of antagonistic allelic effects necessary for complementary gene action. On the other hand, the maintenance of phenotypic similarity despite preceding genetic divergence requires the accumulation and fixation (by stabilizing selection) of antagonistic allelic effects independently within the two species (DeVicente and Tanksley 1993; Mansur et al. 1993; Kim and Rieseberg 1999; Albertson and Kocher 2005).

The approach I took in **Chapter 2** is based on a large literature survey of published articles on plant and animal hybrid systems. I collected quantitative phenotypic data of hybridized species and their offspring from a total of 62 plant and 15 animal systems and calculated frequencies of transgressive segregation and phenotypic differentiation between the parental species pairs. Molecular sequence data from GenBank was used to calculate pairwise sequence differences to obtain an estimate of the genetic distance between the parental species. Transgression frequencies were mapped onto published phylogenetic trees and the comparative method (Felsenstein 1985) was used to control for phylogenetic non-independence before regressing independent contrasts in transgression frequency against contrasts in genetic distance. I found that 43% of transgressive segregation observed in hybrids of eudicot plants was predicted by the genetic distance between the parental species. Although the large majority of species pairs showed a negative correlation between the extent of phenotypic differentiation and the occurrence of transgressive trait expression in their hybrids, the effect of genetic distance on transgression seemed unconfounded by the potentially conflicting effects of phenotypic similarity. Interestingly, the same analysis in animals revealed a significantly negative relationship between transgression frequency and genetic distance. Also in animals, phenotypically more dissimilar pairs had less transgressive offspring but in this data set, I found a strong correlation between phenotypic differentiation and genetic distance. Hence, the unexpected result may be explained by the conflicting effects of genetic and phenotypic divergence on transgression frequency, with the latter outweighing the former in the animal data set. Furthermore, I found that 36% of all plant traits and 29% of all animal traits were transgressive, results that are in agreement with the previous review on transgression frequencies that found 59% and 31% of transgressive traits, respectively (Rieseberg et al. 1999).

In **Chapter 3**, I again tested the prediction that the frequency of transgression in interspecific hybrids is predictable from the degree of genetic divergence between the parental

species, but here I used an experimental approach employing interspecific hybrids of cichlid fish bred in the laboratory. Crosses were made from nine haplochromine cichlid species yielding six different F1 and five different F2 cross types with gradually increasing genetic distances and varying phenotypic similarity. Multi-trait phenotypic variation was quantified using landmark-based geometric morphometrics and thin plate spline statistical methods. Genetic distances were calculated from mitochondrial gene sequences and converted in absolute divergence times using two different relaxed molecular clocks (Genner et al. 2007) and one internally calibrated clock (Sturmbauer et al. 2001), all three specifically developed for African cichlid fish. According to these, crosses in this experiment covered divergence times of between a few thousand years to 2.7/3.8/7.4 million years (MY) depending on the molecular clock used.

The results of this experiment revealed that transgressive segregation is frequent. Extreme trait values were observed in every one of the F1 and F2 crosses generated. The amount of transgression observed in F2 hybrids increased linearly with time since speciation confirming the prediction. In the F1 generation on the other hand, large amounts of transgression were observed in both closely and distantly related crosses with transgression nearly absent at intermediate genetic distances, resulting in a u-shaped relationship between transgression and divergence time. The unexpected large amount of transgression in F1 hybrids of closely related species may be explained by heterosis effects causing an overall increased fitness in the F1 generation, causing more healthy individuals to express larger (transgressive) trait values, but this remains speculative.

The phenotypic differentiation of the parental species was strongly correlated with genetic distance, indicating that genetic and phenotypic divergence of the overall body shape proceeded at similar rates in the nine species used. However, phenotypic dissimilarity did not predict an increase in transgression frequency in this experiment suggesting that the effect of genetic distance was not compromised by the counteracting effects of phenotypic divergence.

In summary, both **Chapter 2 and 3** demonstrate that the magnitude of phenotypic novelty in interspecific hybrids can be predicted from the genetic distance between the parental species, and that novel traits are more likely observed between genetically distant species. These findings have important implications for the role of hybridization in adaptive evolution and are particularly interesting when revising the feasibility of the hybrid swarm origin hypothesis for African cichlid radiations stating that hybridization may facilitate rapid diversification in a newly colonized habitat in response to ecological selection (Seehausen 2004). The widest cross in our experiment represents similar divergence times (at least when estimated from a relaxed molecular clock based on the fragmentation of Gondwanaland plus recent geological events) to those estimated for the hypothesized multiple ancestors of two major cichlid radiations (Lake paleo-Makgadikgadi (Joyce et al. 2005), Lake Victoria (Seehausen et al. 2003). Considering the large amount of transgressive segregation emerging from the most distant cross in this experiment, the potential of hybridization to endow the phenotypic diversification of a group with new momentum becomes more plausible.

Chapter 4 - Investigating the rate at which pre- and postmating incompatibilities accumulate in African cichlid fish.
To relate the adaptive novelty arising in hybrids between species of increasing genetic distance (Chapter 2 and 3) with the actual viability of these crosses, in **Chapter 4**, I investigated the rate at which genetic incompatibilities accumulate with time since speciation in African cichlid fish.

Since the method was introduced in the landmark studies on fruit flies by Coyne and Orr (1989, 1997), experiments have been conducted comparing the degree of premating isolation or postmating incompatibility with interspecific genetic distance in many different groups of animals (Knowlton et al. 1993; Foltz 1997; Sasa et al. 1998; Presgraves 2002; Price

and Bouvier 2002; Lijtmaer et al. 2003; Fitzpatrick 2004; Malone and Fontenot 2008) and plants (Moyle et al. 2004; Scopece et al. 2007; Lowry et al. 2008). So far, such study was lacking in cichlid fish. **Chapter 4** contributes empirical data relevant to outstanding questions in speciation and adaptive radiation research in African cichlid fish, for example, if interspecific hybridization at the onset of radiations between distantly related species (Seehausen 2004) is a realistic scenario when taking into account reproductive biology. Another other question I attempted to answer here is if premating incompatibilities accumulate faster than intrinsic postmating barriers in allopatric species pairs as previously hypothesized (Fisher 1930; Lande 1981; West-Eberhard 1983; Coyne and Orr 2004) and experimentally confirmed in a group of sexually dimorphic fish (Mendelson 2003).

I produced interspecific hybrids between 16 cichlid species pairs, representative of the entire haplochromine cichlid radiation, with gradually increasing genetic distances (spanning a few thousand years to 5-22 MY divergence time) and measured the viability of hybrids at four different life stages, spanning ages from the zygote to adulthood. Hybrid viability wad controlled for the viability found in corresponding homospecific parental crosses. Premating compatibility was measured as spawning latency, i.e. the time that elapsed until spawning occurred. Genetic distances were translated into absolute evolutionary age using molecular clock calibrations, and a weighted node-averaging procedure was used to calculate phylogenetically independent contrasts in incompatibility and in divergence time (Felsenstein 1985). This is the first to use relaxed molecular clock calibrations to translate genetic distances into absolute age for comparing evolutionary rates of different components of reproductive isolation.

I found that premating isolation accumulated faster initially but then changed little with increasing genetic distance between species. It increased by 9/4/2/% per MY divergence time (depending on the molecular clock). In contrast, postmating isolation between closely related species was almost completely absent but then accumulated rapidly causing complete hybrid inviability after 4.4/8.5/18.4 MY. Especially, failure in fertilization (24/11/5% per MY) and larval mortality (29/13/6% per MY) were strongly correlated to the degree of genetic unrelatedness. Hence, the decay of hybrid viability proceeded at a rate two- to three-fold faster than the increase of premating isolation.

These results allow for several insightful conclusions. First, they contradict the prediction that premating isolation and postmating incompatibilities should accumulate at similar rates in allopatry because genetic drift affects every trait type equally (Coyne and Orr 2004). The reason for the observed pattern may lie in the conserved courtship behaviour of widely diverging cichlid fish species (McElroy and Kornfield 1990), which could be responsible for the rather weak mating barriers we found across large genetic distances.

Second, the rate at which strong intrinsic incompatibilities arise in the haplochromine radiation appears to be orders of magnitude lower than rates of speciation that have been suggested within individual lake radiations. Following from this, most of the cichlid species diversity must be maintained by mechanisms other than intrinsic isolation, and is more likely depended on divergent ecological adaptations to the heterogeneous lake habitats, rendering the species flocks of the African Great Lakes highly vulnerable to changes in the environments and habitat destruction.

Third, calibrating the time window for successful hybridization between species is of particular relevance for the cichlid model system because the capacity to produce viable hybrids over large genetic distances has been suggested a key element contributing to the exceptionally species-rich and rapid adaptive radiations of cichlid fish. In my experiment, the maximum crossing distance still allowing for the production of viable hybrids was 4.4/8.5/18.4 my, and ultimately limited by high mortality rates before the free swimming stage. This demonstrates that the production of viable hybrids in cichlid fish does not cease for a long time after lineages have split. The weakness (or absence) of barriers to gene flow

over large genetic distances I observed confirms that hybridization could have theoretically contribute to the rapid diversification of cichlid fish as previously suggested for the radiations of Lake Victoria (Seehausen et al. 2003) and Lake Makgadikgadi (Joyce et al. 2005) and Lake Mweru (Chapter 5, this thesis). Interestingly, some hybrids showed higher survival rates than their parental taxa hinting at heterosis-like effects due to the overall increased heterozygosity in the F1 generation, another factor that may facilitate ecological diversification in cichlid radiations with hybridization background.

Chapter 5 - Phylogenetic and ecomorphological evidence for a newly discovered AfricanGreat Lake cichlid radiation that emerged through hybridization and competition between multiple divergent lineages

Several hypotheses have been proposed to explain the impressive volume of the adaptive radiations of haplochromine cichlid fish in the Great African Lakes (Genner et al. 2004; Kocher 2004) that have evolved in surprisingly short periods of time (Seehausen 2002; Stager and Johnson 2008). The 'evolutionary release hypothesis' argues for a competitive advantage in newly invaded heterogeneous environments (Fryer and Iles 1972). However, it fails to explain why the genetic variation required for ecological speciation does not seem to become exhausted by the presumably strong selection acting during the divergence of species (Grant and Grant 2002). A recently evoked hypothesis provides a solution to this paradox. If distantly related lineages hybridize upon secondary contact in novel or perturbed habitat, they can generate hybrid populations with large variation in quantitative traits providing the opportunity for rapid radiation in response to diversifying selection (Seehausen 2004). In **Chapter 5**, I investigated if this scenario can be applied to a new large cichlid radiation, which had previously gone completely unnoticed, in an isolated African Great Lake that contains several distantly related haplochromine species.

On a fieldtrip to Lake Mweru (Zambia/Democratic Republic of the Congo), we sampled and identified more than 50 phenotypically distinct, putative species with up to 15 at any single collection site, varying in, size, body shape, jaw morphology, male breeding colouration, and habitat use. To understand the evolutionary dynamics and the temporal patterns that led to this cichlid radiation, I reconstructed their phylogeny by sequencing the most variable section (D-loop) of the mitochondrial DNA control region of a large number of individuals (n = 228). This data was combined with published sequences of the closely related palaeo-Makgadikgadi radiation (n = 98), and representatives of all other major African radiations (Lakes Victoria, Malawi, Tanganyika) and riverine haplochromines. To capture the phenotypic diversity of Lake Mweru, I measured 13 morphological distances reflecting ecologically relevant shape variation on 192 individuals. Further, I compared the total morphospace coverage of Lake Mweru's phenotype diversity to that of the classic adaptive radiations of Lakes Victoria, Malawi and palaeo-Makgadikgadi. The divergence between clades and the onset of each radiation was dated with two different, relaxed molecular clocks, one based on cichlid fossil record calibration plus recent biogeographical events, the other on the break up of Gondwanaland plus recent biogeographical events (Genner et al. 2007). Additionally, I applied an internally calibrated, linear clock calibrated to the geology of Lake Malawi (Sturmbauer et al. 2001).

The mitochondrial genealogy revealed that at least eight phylogenetically distinct lineages of haplochromine cichlids must have independently colonized Lake Mweru, which apparently resulted in four different sub-radiations defined by nuclear genomic and phenotypic variation. Molecular clock estimates suggest that endemic species diversity emerged very recently (0.21-0.94 MY), and nearly simultaneously, out of five of these lineages. Strikingly, there is evidence for mitochondrial haplotype capture between all of these five lineages, that had diverged 1.70/1.65/2.94MY, 2.52/3.27/6.32MY, 2.90/4.20/8.34MY, and 3.11/4.72/9.51MY ago (estimates depending on the molecular clock

used). Phylogenetic reconstruction using 1331 AFLP loci revealed, that hybridization explained the presence of distantly related haplotypes in all radiating serranochromine clades of Lake Mweru. Together, the four radiations of Lake Mweru cover as much volume in ecomorphological space as the classic cichlid radiations. Analysis of morphological disparity shows that the four sub-radiations are complementary to each other in morphospace, with each clade occupying a distinct area, suggesting that competitive exclusion acted during their diversification.

These data are the strongest yet available evidence that the pre-existing variation on quantitative trait loci, brought about by hybridization between distantly related lineages, has fuelled the rapid diversification of cichlid species, and that competition between the distant relatives has caused co-evolutionary patterns in the ecomorphology of each sub-radiation.

Chapter 6 – Evidence for ecological speciation: Ecomorphological adaptation but not genetic distance predicts assortative mating between young members of an adaptive cichlid radiation

Another conceptual challenge when trying to understand the dynamics of adaptive radiations is the apparent ease with which new ecotypes originate despite the constant opportunity for homogenizing gene flow in the spatially often constrained habitat dimensions. Usually, at the early stages of radiations, physiological barriers are weak or absent and hybrids between the incipient species are not (yet) compromised by genetic incompatibilities (i.e. hybridization does not infer intrinsic fitness disadvantages). Hence, theories of speciation requiring periods of independent evolution in allopatry (Mayr 1963; Turelli et al. 2001) for the build-up of reproductive isolation that are merely by-products of genetic differences accumulating in diverging populations due to drift (Gavrilets and Boake 1998) and/or selection (Dobzhansky 1951; Coyne and Orr 2004) are not sufficient to explain the rapid emergence of functional diversity during adaptive radiations.

The idea that access to new ecological resources can promote divergence in young stages of adaptive radiations emerged early in the literature (Simpson 1944). Recently, this hypothesis emphasizing the role of divergent natural and sexual selection and the feasibility of 'ecological speciation' has been revitalized (Schluter 2000) and now receives much support from theoretical and empirical investigations. Ecological speciation predicts that premating incompatibility can arise as a byproduct of divergent ecological selection on traits affecting mate choice via pleiotropy (Kilias et al. 1980; Dodd 1989; Vines and Schluter 2006) or through divergent selection directly acting on (female) mating preferences (Endler 1992; Schluter and Price 1993; Boughman 2002; Maan et al. 2006; Seehausen et al. 2008). Theoretical work predicts that assortative mating can then evolve through ecologically-based, reinforcement-like mechanisms that reduce the exogenous fitness of intermediate hybrid genotypes in parental habitat (Kondrashov and Kondrashov 1999; Doebeli and Dieckmann 2003; Gavrilets 2004; Kawata et al. 2007; Leimar et al. 2008).

In **Chapter 6**, I investigated predictors of assortative mating between members of a young cichlid radiation of the genus *Pseudocrenilabrus* in Lake Mweru, Zambia/DRC. On the fieldtrip in 2005, live specimens of two allopatric, phenotypically generalized lineages were collected, that are only distantly related genetically, and are invoked in the colonization of Lake Mweru and hybridization at the onset of the radiation (*Pseudocrenilabrus philander* from Lake Bangweulu and Lake Mweru). Additionally, two endemic species representing phenotypically derived ecotypes were collected from Lake Mweru. The last two are sympatric and closely related to *P. philander* from Lake Mweru. To measure the genetic differentiation of the species pairs tested, I calculated uncorrected p-distances from molecular sequence data. Divergence in ecomorphology and male breeding colouration was quantified using 13 morphometric distances and a colour index scored at 10 different places on body and fins. I conducted mate choice experiments in four pairwise combinations in the laboratory to test i)

the potential for hybridization between unrelated colonists and ii) the evolution of assortative mating during subsequent ecological speciation.

I found both predictions confirmed. The two allopatric generalists mated randomly with each other despite the large genetic distance between them. In contrast, the generalist lineage from Lake Mweru had evolved significant assortative mating against both sympatric, ecologically derived species and both derived species mated assortatively against each other. Phenotypic distance was a good predictor for the strength of assortative mating. Premating isolation increased with ecomorphological dissimilarity and, to a lesser extent, with increasing differences in male colouration. Genetic distance on the other hand did not explain variance in premating isolation.

Together, these data suggest that upon colonization of a new lake, distantly related cichlid lineages may not have any prezygotic mating isolation barriers and are likely to hybridize. Yet, assortative mating preferences may then evolve rapidly when associated with ecological divergence during subsequent adaptive radiation.

Chapter 7- Conclusions

In **Chapter 7**, I summarize the main conclusions of the work presented in this thesis.

First, I confirmed the prediction that genetically more divergent species pairs produce hybrids with a higher frequency of phenotypic novelty. This was confirmed for eudicot plants with a large data set extracted from published articles, and for laboratory-bred hybrids of cichlid fish.

Second, I determined the accumulation rate of reproductive incompatibility in cichlid fish and found that incompatibilities linearly increased with time since speciation. Further, I found that hybrid viability does not cease until 4.4/8.5/18.4 million years divergence time.

Third, I describe the discovery of four large new adaptive radiations of cichlid fish in Lake Mweru, Zambia, that have evolved under the contribution of hybridization between largely divergent lineages and show patterns of coevolution.

Fourth, I found that ecological divergence is a better predictor than genetic divergence for the strength of assortative mating among three young, sympatric members of an adaptive cichlid radiation in Lake Mweru. The absence of premating isolation between two allopatric, distantly related populations demonstrates their potential for hybridization in secondary contact and makes the hypothesized scenario of hybridization with subsequent radiation more likely.

In summary, this thesis provides evidence for the potential of interspecific hybridization to contribute to adaptive evolution and phenotypic diversification.

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Chapter 2

Genetic distance between species predicts novel trait expression in their hybrids

Rike B. Stelkens[1, 2] *, Ole Seehausen[1, 2]

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1. Division of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, Baltzerstr. 6, CH-3012 Bern, Switzerland
2. Department of Fish Ecology and Evolution, Centre of Ecology, Evolution and Biogeochemistry (CEEB), Eawag Swiss Federal Institute of Aquatic Science and Technology, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland

* Corresponding author: email: rike.stelkens@unil.ch; tel: +41 21 692 4249; fax: +41 21 692 4165

ABSTRACT

Interspecific hybridization can generate transgressive hybrid phenotypes with extreme trait values exceeding the combined range of the parental species. Such variation can enlarge the working surface for natural selection, and may facilitate the evolution of novel adaptations where ecological opportunity exists. The number of quantitative trait loci fixed for different alleles in different species should increase with time since speciation. If transgression is caused by complementary gene action or epistasis, hybrids between more distant species should be more likely to display transgressive phenotypes. To test this prediction we collected data on transgression frequency from the literature, estimated genetic distances between the hybridizing species from gene sequences, and calculated the relationship between the two using phylogenetically controlled methods. We also tested if parental phenotypic divergence affected the occurrence of transgression. We found a highly significant positive correlation between transgression frequency and genetic distance in eudicot plants explaining 43% of the variance in transgression frequency. In total, 36% of the measured traits were transgressive. The predicted effect of time since speciation on transgressive segregation was unconfounded by the potentially conflicting effects of phenotypic differentiation between species. Our analysis demonstrates that the potential impact hybridization may have on phenotypic evolution is predictable from the genetic distance between species.

KEYWORDS: adaptive evolution, comparative method, genetic distance, hybridization, transgressive segregation, speciation.

INTRODUCTION

The recombination of genetic material among lineages with divergent evolutionary histories can give rise to novel phenotypes. For more than ten thousand years, since the beginning of domestication of plants and animals, humans have made explicit use of this. Despite influential early publications (Anderson 1949; Anderson and Stebbins 1954; Stebbins 1959; Lewontin and Birch 1966; Stebbins 1966; Templeton 1981) the role of hybridization in evolution, certainly of animals, had for many years received only limited attention by evolutionists. This has recently begun to change. It is now clear that hybridization between species is much more common than was thought previously (Grant and Grant 1992; Arnold 1997; Dowling and Secor 1997; Rieseberg et al. 1999; Barton 2001; Seehausen 2004; Mallet 2007; Schwenk et al. 2008). There is also convincing evidence for that hybridization may facilitate adaptive evolution within species (Grant and Grant 2008) and that it may lead to evolutionary novelty, i.e. to the emergence of novel adaptations and new species, both in plants (Lexer et al. 2003b) and animals (Schliewen and Klee 2004; Gompert et al. 2006; Mavarez et al. 2006). Some adaptive radiations may have been fuelled by hybridization between distantly related species in plants (Barrier et al. 1999), animals (Feder et al. 2003; Seehausen et al. 2003; Joyce et al. 2005; Mallet 2007) and prokaryotes (Vernikos et al. 2007). Much of this paradigm shift has been driven by developments in molecular genetics that made it possible to identify hybrid individuals (Rieseberg and Linder 1999; Anderson and Thompson 2002) and lineages (Ungerer et al. 1998) more easily and track the traces of reticulate evolution with more confidence (Marri et al. 2007).

Next to their intrinsic fitness, the evolutionary potential of hybrid populations depends on the ecological competitiveness of hybrid genotypes. Simulation models (Buerkle et al. 2000) and experiments (Abbott 1992; Jackson and Tinsley 2003; Lexer et al. 2003a) suggest that hybrid populations are likely to persist only if they can occupy previously underutilised fitness peaks on the local adaptive landscape. However, in most cases hybrids resemble one of the parents or express intermediate trait values that lay between the parental means. Intermediate hybrid phenotypes are not likely to persist without spatial isolation from the parents (Barton and Hewitt 1985) unless an underutilized fitness peak requires intermediate trait values (Mallet 2007). Yet, hybrids frequently express trait values exceeding the range between the parental means, which is referred to as transgressive segregation (Slatkin and Lande 1994; Rieseberg et al. 1999). Phenotypes are transgressive if they lie outside the phenotypic range of both parental species. Theoretically, transgressive traits can provide hybrid genotypes with novel adaptive potential, not shared by either parental population. Populations of such hybrid genotypes may then diverge from the parental species through the same mechanisms that play a role in classical ecological speciation (Seehausen 2004). Ecological hybrid speciation facilitated by transgressive segregation has been demonstrated in detail in hybrid sunflower species (Schwarzbach et al. 2001; Lexer et al. 2003b; Rieseberg et al. 2003).

Transgressive segregation is common and widespread. Rieseberg at al. (1999) found evidence for transgressive segregation in 110 of 113 studies on hybridizing plant species, and in 45 of 58 cases of hybridizing animal species. They further found that 59% of the 579 investigated traits in plants, and 31% of the 650 traits in animals, were transgressive. Several different mechanisms have been proposed to explain how the rearrangement of genomes can create phenotypic novelty (Rick and Smith 1953; Grant 1975; DeVicente and Tanksley 1993; Monforte et al. 1997; Rieseberg et al. 1999). A widely accepted view is that transgression is the result of the recombination of alleles at quantitative trait loci (QTL), that are fixed for alleles of opposite sign in the parents which sum up to an extreme trait value when recombined in their hybrids a mechanism commonly referred to as complementary gene action. While parental phenotypes are constrained to a certain trait value range (because each

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parent fixed counteracting alleles at different QTLs for the same trait), some of their hybrids can inherit complementary alleles from both parents, generating transgressive hybrid phenotypes. Although non-additive effects by overdominance (in which the combination of divergent alleles at a particular locus endows the heterozygote with a more extreme trait value than both homozygotes) and epistatic interactions (the action of one gene is modified by one or several other genes) may contribute, quantitative genetic studies on plant hybrids consistently identified complementary gene action as the primary cause of transgression (Weller et al. 1988; De Vicente and Tanksley 1993; Mansur et al. 1993; Clarke et al. 1995; Ecke et al. 1995; Li et al. 1995; Monforte et al. 1997; Bradshaw et al. 1998; Kim and Rieseberg 1999; Rieseberg et al. 2003).

Given a purely additive regime, transgression due to complementary gene action can only be observed in the F2 and higher hybrid generations. In the F1 generation, additive effects only produce intermediate phenotypes. However, if dominance prevails at some loci contributing to complementary gene action, transgressive phenotypes can already occur in F1 hybrids. Dominance produces extreme trait values in the F1 generation because hybrid individuals express only the dominant allele at all heterozygous loci, and so end up expressing fewer alleles with antagonistic effects on different loci than their homozygous counterparts. For this, parental species must be recessive homozygotes for at least one locus, and it must be a different locus in each parental species (e.g. the diploid two-locus two-allele parental genotypes A_bb and aaB_ (each with trait values of 0) can produce A_B_ or aabb F1 hybrids with transgressive trait values of +2 or -2, respectively).

We predicted that if some of the transgression in interspecific F1 hybrids is caused by complementary gene action or epistasis, its frequency should correlate positively with the genetic distance between hybridizing species, because the number of loci at which two different species have fixed alleles with opposite sign should increase with time since speciation. To test this we collected data on the frequency of transgressive segregation in hybrids from published work, and molecular sequence data for the same species from GenBank. We calculated pairwise sequence differences between hybridizing species. We then mapped these and transgression frequency on published phylogenetic trees. Finally we calculated independent contrasts (Felsenstein 1985) in genetic distance and in transgression frequency between pairs of hybridizing species for a test of the predictions that is controlled for phylogenetic non-independence.

Variation in the extent of phenotypic differentiation between the parental lines can potentially confound the predicted relationship between genetic distance and transgression. Phenotypically similar species are more likely to produce transgressive hybrid offspring than dissimilar species. This is because the maintenance of phenotypic similarity despite proceeding genetic divergence requires the accumulation and fixation (by stabilizing selection) of antagonistic allelic effects independently within the two species (DeVicente and Tanksley 1993; Mansur et al. 1993; Kim and Rieseberg 1999). Hence, two similar species that have experienced stabilizing selection on the same traits are likely to eventually fix different alleles at some QTLs, which would then cause transgression when these are recombined in hybrids. Conversely, phenotypically divergent species are less likely to produce transgressive offspring as the genetic basis for complementary gene action may be missing because of the fixation of alleles with opposite signs on loci with a consistent directional selection history. To test if phenotypic divergence, besides genetic distance, also affected the occurrence of transgression, we calculated an index of parental phenotypic divergence for each of the traits included in our analysis.

METHODS

Literature Search

All cases used in our analysis were identified in a search using Web of Science (http://portal.isiknowledge.com/portal.cgi) with the keyword combination "interspecific hybrid* AND morpholog*" (965 hits). From this literature we selected studies that met the following criteria: (1) Finding transgressive traits for breeding purposes was not the aim of the study. (2) Data from wild hybrids were included only if their hybrid identity was confirmed with molecular markers. (3) Data were present for at least three different traits. Trait ratios (e.g. leaf width / leaf length) were excluded, except if neither numerator nor denominator were included separately. (4) Data had to be quantitative. We excluded qualitative data (e.g. illustrations of leaf shapes, description of flower colouration). (5) To obtain a comparable measure of genetic distance between species, we used the same gene for all species. This required availability of sequence data on NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) (for details see below). (6) Sequences had to be longer than 500 bp.

A total of 62 plant hybrid systems met our criteria, comprising a multitude of taxa (47 different eudicot crosses, 12 monocot crosses, 2 crosses within magnoliids and one within *Nymphaeaceae*; Table 1; Supplementary Fig. 1). In addition, we were able to collect a small data set on animal species comprising 15 hybrid systems, of which 12 were Teleost fish, of which again 8 crosses within the Teleost family *Cyprinidae*.

We first ran all analyses exclusively on F1 before including the cases where only data on BC (hybrids backcrossed to one or both of the parents), F2, F3 or wild hybrids were available (Table 1). The detectability of transgression in our analysis therefore was mainly limited to cases involving loci with heterozygous effects or dominant alleles, the complementation among which is visible in the heterozygous F1 hybrids.

Assessment of transgression frequency

We collected all available phenotypic data from published articles, including morphological, physiological and life history traits of both parental species and their hybrid offspring. Character means that lay outside the range between the means of both parental species in a negative or positive direction were defined as transgressive. Where only phenotypic ranges were given, but no mean values, we considered hybrids as transgressive if part of their trait value range fell outside of the combined parental ranges. Where means and ranges were given, we only scored those traits as transgressive that had hybrid means outside of the range of the two parental means, regardless of the distribution of the trait ranges, which is conservative with regard to our expectation. Hybrid means can fall between the parental means while the hybrid range can still exceed the parental trait range. We then calculated the ratio between the number of traits that were transgressive to the total number of traits that were measured (hereafter this ratio will be referred to as 'transgression frequency').

Table 1: The 62 plant and 15 animal hybridized species pairs with genetic distances (uncorrected p-distances calculated from ribosomal DNA sequences ITS 1 and 2 for plants and from cyochrome b for animals), transgression frequencies, number of phenotypic traits assessed, hybrid generation, and the source reference.

hybrid system	genetic distance	trans-gression frequency	n traits	hybrid gene-ration	source reference
PLANTS					
Rosa rubiginosa x R. sherardii	0.032	1	5	F1	G. Werlemark, H. Nybom, Hereditas, 134, 1 (2001)
Rosa sherardii x R. villosa	0.028	0.6	5	F1	G. Werlemark, H. Nybom, Hereditas, 134, 1 (2001)
Trifolium alexandrinum x T. constantinopolitanum	0.009	0.0909	11	F1	A.K. Roy et al, Plant Cell Report, 22, 9 (2004)
Trifolium alexandrinum x T. resupinatum	0.053	0.6667	6	F1	P. Kaushal et al, Plant Cell Tissue and Organ Culture, 83, 2 (2005)
Trifolium repens x T. ambiguum	0.022	0.0667	15	F1	M.T. Abberton et al, Plant Breeding, 117, 5 (1998)
Medicago sativa sativa x M. falcata	0.007	0.2778	18	F1	H. Riday, personal communication
Medicago sativa x M. rugosa	0.028	0.6667	3	F1	Y. Mizukami et al, Plant Cell Tissue and Organ Culture, 84 (2006)
Medicago sativa x M. arborea	0.0245	0.4667	15	F1	E. Nenz et al, Theoretical and Applied Genetics, 93 (1996)
Pachyrhizus tuberosus x P. ahipa	0.0768	0.75	16	F1	W.J. Gruneberg et al, Genetic Resources and Crop Evolution, 50, 7 (2003)
Lotus alpinus x L. conimbricensis	0.088	0.3636	11	F1	L.S. O'Donoghue et al, Canadian Journal of Botany, 68 (1990)
Lotus burtii x L. ornithopodioides	0.074	0.2727	11	F1	L.S. O'Donoghue et al, Canadian Journal of Botany, 68 (1990)
Phaseolus vulgaris x P. acutifolius	0.0628	0.5	6	F3	S. Honma, Journal of Heredity, 47 (1956)
Phaseolus vulgaris x P. lunatus	0.0727	0.75	4	unclear	S. Honma, O. Heeckt, Journal of Heredity, 50 (1959)
Populus trichocarpa x P. deltoides	0.0085	0.2	25	F1	R. Wu et al, American Journal of Botany, 84, 2 (1997)
Populus trichocarpa x P. deltoides	0.0085	0.2083	8	F1	R. Wu, R.F. Stettler, Heredity, 81 (1998)
Cucumis sativus x C. hystrix	0.04	0	11	F1	J.F. Chen et al, Euphytica, 96, 3 (1997)
Cucumis sativus x C. hystrix	0.04	0	14	F1	J.F. Chen et al, Canadian Journal of Botany, 82 (2004)
Brassica juncea x B. rapa (variety toria)	0.012	0.625	8	F2	B.R. Choudhary et al, Plant Breeding, 21, 4 (2002)
Brassica juncea x B. rapa (yellow sarson)	0.012	0.75	8	F2	B.R. Choudhary et al, Plant Breeding, 21, 4 (2002)
Brassica rapa x Brassica napus	0.0382	1	6	F1	L. Changming et al, Sabrao Journal of Breeding and Genetics, 33, 2 (2004)
Carica papaya x Vasconcellea quercifolia	0.156	0.2857	7	F1	R.A. Drew et al, Aust. J of Exp. Agriculture, 46 (2006) R.L. Stokoe et al, Annals of Botany, 88, 4 (2001)
Eucalyptus acmenoides x E. cloeziana	0.0204	0.3333	3	F1 (wild)	

Cross	Value1	Value2	N	Type	Reference
Eucalyptus erythronema x E. stricklandii	0.0204	0.32	25	F1	K. Delaporte et al, Scientia Horticulturae, 89, 1 (2001)
Lagerstroemia indica x L. speciosa	0.049	0	5	F1	C. Pounders et al, HortScience 42, 6 (2007)
Helianthus annuus x H. debilis cucumerifolius	0.013	0.1667	11	BC	S.C. Kim, L.H. Rieseberg, Genetics, 153, 2 (1999)
Helianthus annuus x H. petiolaris	0.003	0.3333	6	BC	C. Lexer et al, Evolution, 57, 9 (2003)
Helianthus annuus x H. salicifolius	0.006	0.4118	17	F1	J. Encheva, M. Christov, HELIA, 29, 45 (2006)
Helianthus annuus x H. tuberosus	0.008	0.4707	17	F1	J. Encheva et al, HELIA, 26, 39 (2003)
Senecio vulgaris x S. squalidus	0.0327	0.5385	26	wild	A.J. Lowe et al, American jJournal of Botany, 83, 10 (1996)
Senecio vulgaris x S. squalidus	0.0327	0.5526	38	wild	J.A. Irwin, R.J. Abbott, Heredity, 69 (1992)
Lycopersicon esculentum x L. pimpinellifolium	0.0105	0.25	8	F1	A.J. Montforte et al, Theoretical and Applied Genetics, 95 (1997)
Lycopersicon esculentum x L. cheesmanii	0.0023	0.125	8	F1	A.J. Montforte et al, Theoretical and Applied Genetics, 95 (1997)
Lycopersicon esculentum x L. peruvianum	0.0291	0.1667	6	F1	S. Doganlar et al, Euphytica, 95 (1997)
Solanum melongena x S. macrocarpon	0.03	0.4737	19	F1	F. Bletsos et al, Scientia Horticulturae, 101, 1-2 (2004)
Solanum commersonii x S. tuberosum	0.073	0.5	8	F1	T. Cardi, Euphytica, 99, 1 (1998)
Solanum commersonii x S. tuberosum	0.073	0.8571	7	F1	F. Esposito et al, Journal of Agriculture and Food Chemistry, 50 (2002)
Solanum torvum x S. melongena	0.051	0	4	F1	K.R. McCammon, S. Honma, HortScience, 18, 6 (1983)
Solanum torvum x S. melongena	0.051	0.5	12	F1	F.A. Bletsos et al, Plant Breeding, 117 (1998)
Solanum acaule x S. tuberosum	0.096	0.5556	9	F1	V.-M. Rokka et al, Plant Cell Reports, 18 (1998)
Coffea liberica x C. canephora	0.0263	0.1667	12	BC	N. Amidou et al.Genetic Resources and Crop Evolution, 54, 5 (2007)
Mimulus lewisii x M. cardinalis	0.002	0.25	12	F2	H.D. Bradshaw et al, Genetics, 149, 1 (1998)
Trichostma lanatum x T. arizonicum	0.028	0.2	5	F1	B.L. Dunn and J.T. Lindstrom, Hortscience, 43 (2), 2008
Trichostma lanatum x T. purpusii	0.036	0.4	5	F1	B.L. Dunn and J.T. Lindstrom, Hortscience, 43 (2), 2008
Gilia capitata capitata x G. capitata chamissonis	0.0118	0	4	F2	E. Nagy, Evolution, 51, 5 (1997)
Dianthus giganteus x D. carthusioanorum	0.011	0.4667	15	F1	S.Y. Lee et al, Scientia Horticulturae, 105, 1 (2005)
Cerastium alpinum x C. glomeratum	0.004	0.1852	27	wild	A.R. Hagen et al, Plant Systematics and Evolution, 230, 3-4 (2002)
Amaranthus retroflexus x A. cruentus	0.0155	0	4	wild	V. Lanta et al, Plant Soil and Environment, 49, 8 (2003)
Oryza sativa x O. glaberrima	0.0105	0.4444	9	BC	G. Aluko et al, Theoretical and Applied Genetics, 109, 3 (2004)
Oryza sativa japonica x O. rufipogon	0.011	0.375	8	BC	P. Moncada et al, Theoretical and Applied Genetics, 102 (2001)
Zea mays x Z. diploperennis	0.2472	0.1818	11	F1	G. Srinivasan, J.L. Brewbaker, Maydica, 44, 4 (1999)
Hordeum vulgare x H. spontaneum	0.0078	1	5	F2	U. Vega K.J. Frey, Euphytica, 29 (1980)
Sorghum bicolor x S. macrospermum	0.132	0	4	F1	H.J. Price et al, Australian Journal of Botany, 53, 6 (2005)
Carex castanea x C. arctata	0.008	0.1538	13	F1 (wild)	M.J. Waterway, Canadian Journal of Botany, 72, 6, (1994)
					Y. Nomura et al, Scientia Horticulturae, 95, 3 (2002)
Allium chinense x A. schubertii	0.285	0.4545	11	F1	

Allium thunbergii x A. caeruleum	0.1615	0.6364	11	F1	Y. Nomura et al, Scientia Horticulturae, 95, 3 (2002)
Allium thunbergii x A. nutans	0.1265	0.1818	11	F1	Y. Nomura et al, Scientia Horticulturae, 95, 3 (2002)
Cypripedium candidum x C. nubescens	0.012	0.2069	29	wild	K. Klier et al, Journal of Heredity, 82, 4 (1991)
Vanilla planifolia x V. aphylla	0.159	0	5	F1	M. Divakaran et al, Scientia Horticulturae, 2006
Lilium nobilissimum x L. regale	0.0893	0.4	5	F1	Y. Obata, Scientia Horticulturae, 84, 1-2 (2000)
Piper nigrum x P. barberi	0.0906	0.6667	12	F1	B. Sasikumar et al, Journal of Horticultural Science & Biotechnology, 74, 1 (1999)
Piper nigrum x P. attenuatum	0.0906	0.3333	12	F1	B. Sasikumar et al, Journal of Horticultural Science & Biotechnology, 74, 1 (1999)
Nuphar microphylla x N. variegata	0.03	0	15	wild	D.J. Padgett, American Journal of Botany, 85, 10 (1998)

ANIMALS

Rutilus rutilus x Abramis brama	0.1233	0	5	wild	A.B. Wood, D.R. Jordan, Journal of Fish Biology, 30 (1987)
Notemigonus crysoleucas x Scardinius erythrophthalmus	0.0332	0.3421	37	F1	N.M. Burkhead, J.D. Williams, Transactions of the American Fisheries Society, 120 (1991)
Leuciscus cephalus x Chalcalburnus chalcoides	0.1271	0	38	wild	B. Ünver, F. Erk'Akan, Journal of Fish Biology, 66 (2005)
Leuciscus cephalus x Chalcalburnus chalcoides	0.1271	0.3438	32	wild	P.S. Economidis, A.I. Sinis, Journal of Fish Biology, 32 (1988)
Semotilus atromaculatus x Campostoma anomalum	0.1905	0	10	F1	M.R. Ross, T.M. Cavender, Copeia, 1981, 2 (1981)
Semotilus atromaculatus x Nocomis biguttatus	0.18	0	7	F1	M.R. Ross, T.M. Cavender, Copeia, 1981, 2 (1981)
Semotilus atromaculatus x Rhinicthys atratulus	0.196	0.4	10	F1	M.R. Ross, T.M. Cavender, Copeia, 1981, 2 (1981)
Notropis spiloperus (Cyprinella spiloptera) x N. whipplei (C. whipplei)	0.1302	0.619	21	F1	N.A. Neff, G.R. Smith, Systematic Zoology, 28, 2 (1979)
Lepomis cyanellus x L. macrochirus	0.1803	0.1765	17	F1	N.A. Neff, G.R. Smith, Systematic Zoology, 28, 2 (1979)
Pleuronectes ferrugineus x P. americanus	0.0775	0.3333	9	F1	I-S. Park et al, Aquaculture Research, 34 (2003)
Salvelinus confluentus (Oncorrhynchus tshawytsha) x S. fontinalis	0.134	0.6	10	wild	R.F. Leary et al, Systematic Zoology, 32, 4 (1983)
Cottus bairdi x C. cognatus	0.0373	0.3846	13	wild	R.E. Strauss, American Midland Naturalist, 115, 1 (1986)
Passerina cyanea x P. amoena	0.0387	0.4	5	wild	M.C. Baker, M.S. Johnson, The Auk, 115, 2 (1998)
Dendroica magnolia x D. coronata coronata	0.089	0.25	4	wild	S.C. Latta et al. The Auk, 115, 2 (1998)
Peromyscus maniculatus x P. polionotus	0.0504	1	4	F1	W.D. Dawson, Evolution, 19, 1 (1965)

Assessment of genetic distance

To obtain genetic distances for all parental species pairs, uncorrected p-distances (Takahashi and Nei 2000; Nei and Kumar 2003) were calculated from gene sequences taken from NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Calculating genetic distances on the basis of other substitution models (e.g. Jukes-Cantor, Kimura 2- parameter) did not affect the results of our analyses.

As the common currency for measuring interspecific divergence (Chapman and Burke 2007) we used the internal transcribed spacer region (ITS I and II) for plants and cytochrome b for animals. Between 1 and 10 sequences per parental species (depending on their availability on GenBank) were aligned in ClustalW (Thompson et al. 1997) and alignments were manually optimised. Genetic distances were calculated in MEGA 4 (Kumar et al. 2004). If multiple sequences available were for a pair of species, we calculated the average of all possible pairs of sequences. In four cases (*Eucalyptus, Dianthus, Cerastium* and *Piper*), where sequences for one of the two parental species of a cross were missing, we calculated the average genetic distance between the available parental species and all other species of the genus for which sequences were available. Further, to test if these averaged distances affected our tests, we re-calculated all analyses without these four taxa and compared the results to those of the complete data set.

Chi-square tests of homogeneity of base pair frequencies calculated in PAUP* 4.0b10 (Swofford 2001) revealed no significant heterogeneity between the hybridized species pairs (p > 0.05 in all cases).

Assessment of phenotypic differentiation

Phenotypic differentiation was calculated by dividing the absolute trait value difference between the two parental species of any cross by the larger of the two trait values, resulting in an index ranging from 0 (no trait differentiation) to 1 (large trait differentiation). This was done for each trait reported per hybridized species pair. Logistic regression was used to test transgressive segregation as binary response variable against differentiation index, running a separate regression analysis for each hybrid system. A one-sample t-test on the slopes from all regression lines was used to assess if they significantly differed from zero.

We also tested if genetic distance was correlated to the degree of phenotypic differentiation by calculating linear regressions of the phenotypic differentiation index of all traits across all hybrid systems against genetic distance.

Calculating Independent Contrasts

Independent contrasts (Felsenstein 1985; Pagel 1999) in p-distances and in transgression frequency were calculated for all pairs of hybridized species (note that the terminal taxa in this analysis are pairs of species, rather than species) and for all superior nodes deeper in the phylogeny down to the pair of nodes right above the last common ancestor of two species pairs. We then used standard regression techniques in JMP 7 (SAS Institute) to estimate the relationship between transgression frequency and genetic distance. This method is equivalent to the phylogenetically weighted averaging procedure that has been used in similar contexts (Fitzpatrick 2002; Bolnick and Near 2005). This procedure is required to control for any phylogenetic inertia in transgression frequency. At the same time it ensures the statistical independence of data points (Harvey and Pagel 1991). Phylogenies were taken from The Angiosperm Phylogeny Group (The Angiosperm Phylogeny Group 2003) and from the Tree of Life project (Maddison and Schulz 1996-2007) (Supplementary Fig. 1).

In the regression analysis, we used Δ genetic distance as the independent variable and Δ transgression frequency as the dependent variable. The data were standardized, i.e. each variable was centred to mean zero by subtracting the mean and dividing by the standard

deviation. Both variables were normally distributed, confirmed with Shapiro-Wilkinson tests for normality. The y-intercept of all regression lines was constrained to the origin. This was necessary because when calculating contrasts, the direction of subtraction between the two values of any variable is random and hence the sign of the contrast is arbitrary as long as the contrasts in the two variables that are tested are calculated by subtraction in the same direction (Garland et al. 1992).

For plants, we used six different levels of phylogenetic inclusiveness in our analysis, gradually climbing down the phylogenetic tree from the tips to the root. The first regression (regression I) contained only contrasts calculated between species within genera (e.g. *Trifolium alexandrinum* x *T. resupinatum* versus *Trifolium repens* x *T. ambiguum*). If a specific hybrid cross was studied in more than one publication, we calculated the average of the transgression frequencies from all studies before applying contrasts. The second regression (regression II) contained all within-genus contrasts again, plus contrasts calculated between genera within families (e.g. within *Fabaceae*: *Trifolium* versus *Medicago*). All genetic distances and transgression frequencies were averaged within genera before calculation of the contrasts. We did not perform a separate analysis on the between-family within-order level because only in two eudicot and two monocot cases did we have data on more than one family within an order, which added only little extra information to the previous regression analysis. The third regression (regression III) hence contained the within-genus and within-family contrasts plus contrasts calculated between orders within the next 'supraordinal' clades (e.g. within *Fabids: Fabales* versus *Malphigiales*). Again, all values were averaged beforehand within orders. The fourth regression (regression IV) was calculated as described above containing all previously calculated contrasts plus contrasts calculated within the next more inclusive taxonomic grouping deeper down towards the root of the tree (e.g. within *Rosids*: *Fabids* versus *Malvids*). The same procedure was applied to calculate the fifth regression containing all contrasts within eudicots (regression V) and monocots, respectively. The sixth regression (regression VI) contained all contrasts within angiosperms. No suitable data was available for gymnosperms.

For animals we used the same taxonomic levels of analysis with the difference that contrasts were only available for regressions II, III and VI.

Finally, to test whether transgression frequency was affected by the number of phenotypic traits reported, we conducted a regression analysis of transgression frequency on the total number of traits.

RESULTS

Analysis of the 62 plant studies examined here, reporting on phenotypic variation in segregating hybrid populations and their respective parental populations, resulted in 36% transgressive traits (249 traits out of a total of 687 traits). An earlier study found as much as 59% transgressive traits in a large survey on plant hybrid systems (Rieseberg et al. 1999).

Analysis of the 15 animal studies resulted in 29% transgressive traits (65 traits out of a total of 222 traits). This frequency of transgression is in close agreement with an earlier study that found 31% transgressive traits in animal hybrids (Rieseberg et al. 1999). Only 14 % of the studies analysed by us where also included in that earlier study, while 86% of our data were not analysed in this way before.

There was no correlation between the number of traits reported and the proportion of transgressive traits within either eudicots ($R^2 = 0.01$, $F_{1,46} = 0.24$, $p = 0.63$), monocots ($R^2 = 0.0$, $F_{1,11} = 0.06$, $p = 0.81$), animals ($R^2 = 0.0$, $F_{1,14} = 0.0$, $p = 0.99$), or the combined data set ($R^2 = 0.01$, $F_{1,77} = 0.56$, $p = 0.46$).

The frequency of transgressive traits increased significantly with increasing genetic distance in eudicot plants. The relationship was particularly strong in the phylogenetically

least inclusive comparisons, when only contrasts between pairs of species within genus were considered (regression I, only F1 hybrids: $R^2 = 0.57$, $F_{1,17} = 21.5$, $p < 0.001$; all hybrids: $R^2 = 0.43$, $F_{1,26} = 18.72$, $p < 0.001$; Fig. 1a). Contrasts from one study were excluded from this analysis because they represented outliers from the distribution (i.e. they fell outside of the upper and lower quartile ± 1.5 * interquartile range). In this study, Bletsos *et al.* (Bletsos et al. 2004) produced interspecific hybrids between the eggplant species *Solanum melongena* and *S. macrocarpon*. Two of the three contrasts in genetic distance between this species pair and other *Solanum* crosses, were unusually high while the associated contrasts in transgression frequency were low (genetic distance / transgression frequency: -0.062 / -0.111 and -0.083 / -0.305). When this study was included, the predictive power of genetic distance decreased but the regression slope remained highly significant (regression I, only F1 hybrids $R^2 = 0.5$, $F_{1,20} = 18.92$, $p < 0.001$).

Interestingly, when contrasts between more inclusive nodes in the phylogeny of angiosperms (regressions II-VI) were included, the signal became successively weaker. The fit between transgression frequency and genetic distance was slightly less tight when contrasts between genera of the same family were added (regression II, only F1 hybrids: $R^2 = 0.41$, $F_{1,24} = 15.78$, $p < 0.001$; all hybrids $R^2 = 0.28$, $F_{1,43} = 14.48$, $p < 0.001$; Fig. 1b). The signal decreased further when contrasts between orders were added (regression III, only F1 hybrids: $R^2 = 0.30$, $F_{1,33} = 13.06$, $p = 0.001$; all hybrids: $R^2 = 0.25$, $F_{1,48} = 14.95$, $p < 0.001$; Fig. 1c), and then remained little changed when contrasts between 'supraordinal' clades (regression IV, only F1 hybrids: $R^2 = 0.24$, $F_{1,37} = 16.76$, $p < 0.001$; all hybrids: $R^2 = 0.27$, $F_{1,54} = 19.95$, $p < 0.001$; Fig. 1d) and contrasts within all eudicots (regression V, only F1 hybrids: $R^2 = 0.21$, $F_{1,40} = 17.64$, $p < 0.001$; all hybrids: $R^2 = 0.28$, $F_{1,57} = 21.45$, $p < 0.001$; Fig. 1e) were added. When we added monocots and analysed all contrasts within angiosperms, i.e. including contrasts from all taxonomic levels of both eudicots and monocots, the signal was abruptly lost altogether (regression VI, only F1 hybrids: $R^2 = 0.11$, $F_{1,51} = 2.04$, $p = 0.158$; all hybrids: $R^2 = 0.00$, $F_{1,76} = 0.08$, $p = 0.77$; Fig. 1f).

Inclusion or exclusion of the four crosses, where sequences for one of the two parental species of a cross were missing and for which we calculated averaged genetic distances between the available parental species and all other species of the respective genera *(Eucalyptus, Dianthus, Cerastium, Piper)*, had little effect on the results (results shown only for F1 hybrids after excluding *Eucalyptus, Dianthus, Cerastium* and *Piper*: regression I, $R^2 = 0.58$, $F_{1,16} = 20.32$, $p = 0.001$; results of regression II and III remained unchanged, regression IV, $R^2 = 0.35$, $F_{1,36} = 18.62$, $p < 0.001$; regression V, $R^2 = 0.34$, $F_{1,37} = 18.33$, $p < 0.001$; regression VI, $R^2 = 0.12$, $F_{1,47} = 1.96$, $p = 0.168$). Hence, the averaging of genetic distance within genera did not bias our results.

The monocot data gave different results. The slopes of almost all regressions were negative but none was significant (regression I, only F1 hybrids: $R^2 = -0.25$, $F_{1,3} = 0.67$, $p = 0.49$; all hybrids: $R^2 = -0.04$, $F_{1,4} = 0.12$, $p = 0.75$; regression II, only F1 hybrids: $R^2 = -0.25$, $F_{1,4} = 1.0$, $p = 0.39$; all hybrids: $R^2 = -0.26$, $F_{1,11} = 3.53$, $p = 0.09$; regression IV, only F1 hybrids: $R^2 = -0.41$, $F_{1,6} = 3.5$, $p = 0.12$; all hybrids: $R^2 = -0.22$, $F_{1,13} = 2.66$, $p = 0.128$; regression V, only F1 hybrids: $R^2 = -0.02$, $F_{1,9} = 0.14$, $p = 0.71$; all hybrids: $R^2 = -0.19$, $F_{1,16} = 2.97$, $p = 0.11$; regression VI, only F1 hybrids: $R^2 = 0.02$, $F_{1,10} = 0.16$, $p = 0.7$; all hybrids: $R^2 = -0.17$, $F_{1,19} = 2.97$, $p = 0.1$). Regression III could not be calculated because our data set contained no monocot hybrid crosses for contrasts between orders of the same 'supraordinal' clade.
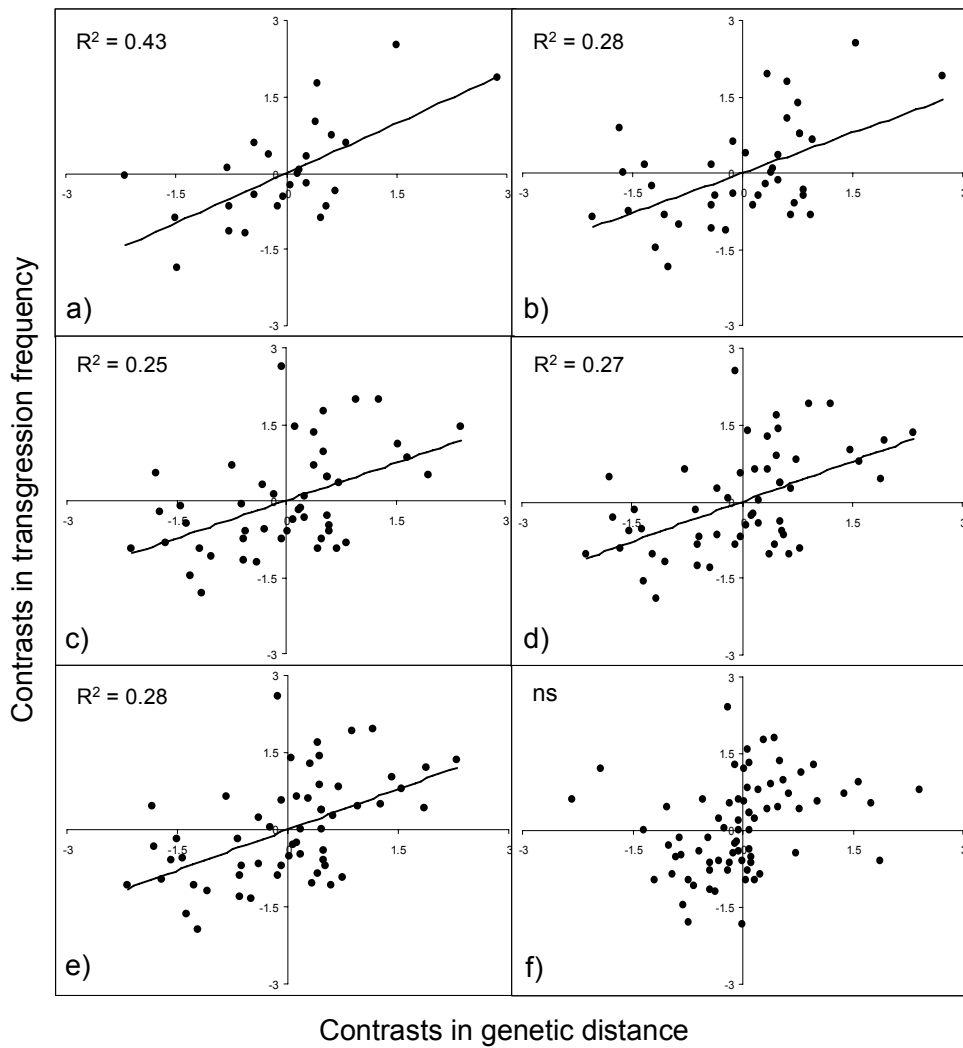
Fig. 1 a-f: Linear regressions (I-VI) of transgression frequency on genetic distance (uncorrected p-distance calculated from internal transcribed spacer region I and II sequences) using the eudicot data set. Independent contrast a) between pairs of species within the same genus; b) same as (a) plus contrasts between genera of the same family; c) same as (b) plus contrasts between orders of the same supraordinal clade; d) same as (c) plus contrasts between supraordinal clades within the next higher taxonomic grouping; e) same as (d) plus contrasts within eudicots; f) same as (e) plus contrasts within angiosperms including eudicots and monocots.

Surprisingly, the animal data set produced significant negative slopes at all levels of phylogenetic inclusiveness (regression II, only F1 hybrids: $R^2 = -0.58$, $F_{1,10} = 12.11$, $p = 0.007$, all hybrids: $R^2 = -0.45$, $F_{1,22} = 16.79$, $p < 0.001$, Fig. 2a; regression III, only F1 hybrids: $R^2 = -0.40$, $F_{1,14} = 8.8$, $p = 0.011$, all hybrids: $R^2 = -0.30$, $F_{1,32} = 8.23$, $p = 0.007$, Fig. 2b; regression VI, only F1 hybrids: $R^2 = 0.47$, $F_{1,15} = 12.33$, $p = 0.004$, all hybrids: $R^2 = -0.21$, $F_{1,35} = 8.95$, $p = 0.005$, Fig. 2c).
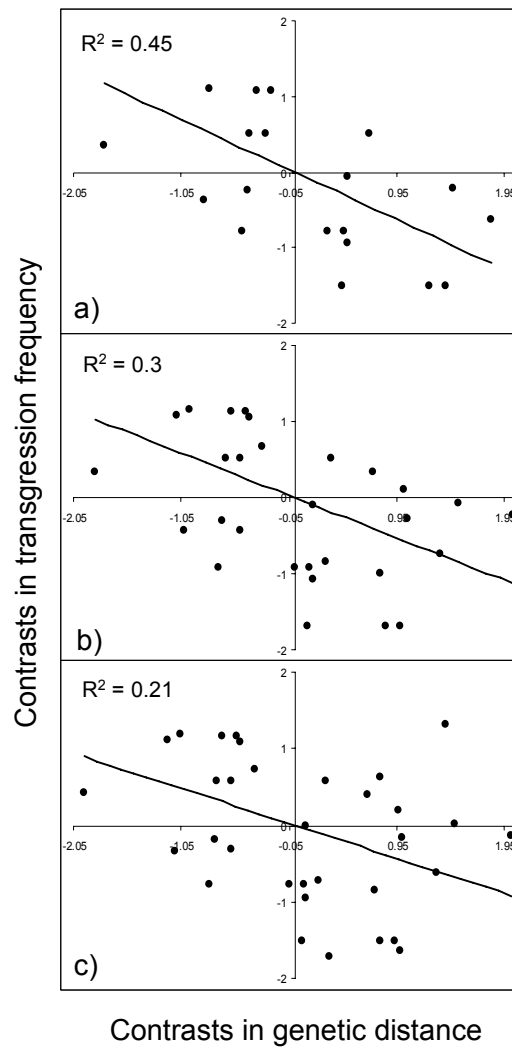
Fig. 2 a-c: Linear regressions (II, III, VI) of transgression frequency on genetic distance (uncorrected p-distance calculated from cytochrome b sequences) using the animal data set. Independent contrast a) between genera of the same family; b) same as (a) plus contrasts between orders of the same class; c) same as (b) plus contrasts between classes within phylum.

In plants, 41 hybrid systems out of 59 (3 systems were excluded here because phenotypic data was only provided as range and not as mean in the source paper) showed a negative correlation between the phenotypic trait differentiation of the parental species and the occurrence of transgression in hybrids (Fig. 3a). Twelve of these 41 negative regression lines were significant, of which four remained significant after sequential Bonferroni correction. Ten systems had regression lines equal to zero and only eight systems showed positive trends of which none was significant. In animals, 10 hybrid systems out of 15 showed a negative correlation between phenotypic differentiation and transgression frequency, four of which were significant (Fig. 3b). None of the animal systems showed a positive trend. A one-sample t-test revealed that, on average, the slopes were significantly different from zero (plants: $t_{59} = -5.04$, $p < 0.001$; animals: $t_{15} = -2.29$, $p = 0.038$).
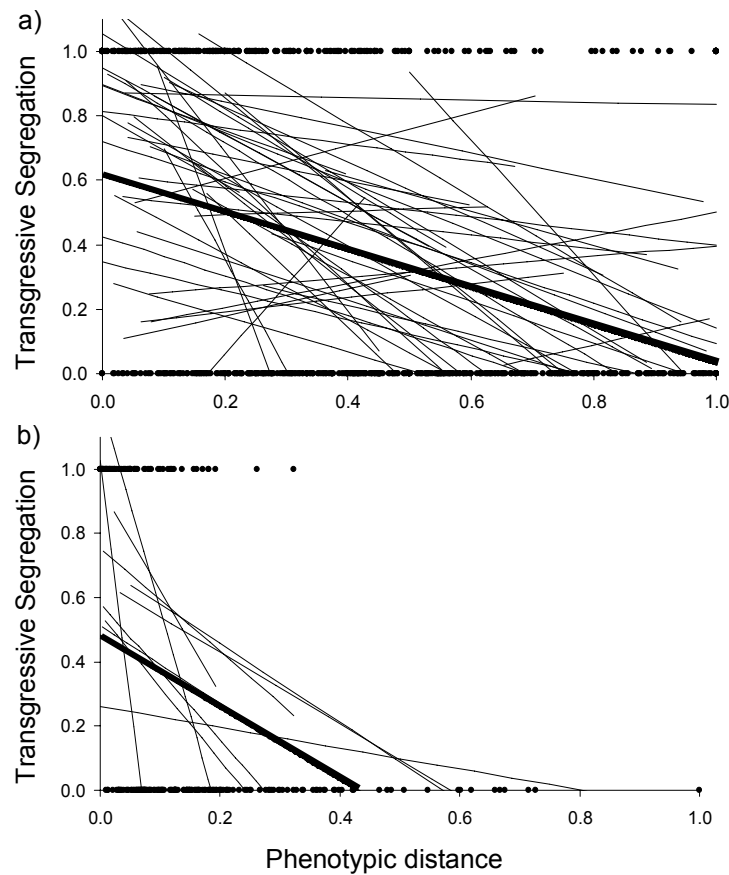
Fig. 3 a-b: Logistic regression of occurrence of transgressive segregation against phenotypic differentiation between hybridizing species of a) plants and b) animals. Each regression line represents one pair of hybridizing species. Sample sizes are n = 55 for plants and n = 15 for animals. The thick line shows the average relationship measured across all traits of all hybrid systems.

In the animal data set, the phenotypic differentiation of the parental species increased significantly with genetic distance ($R^2 = 0.7$, $F_{1,219} = 17.86$, $p < 0.001$) using phenotypic data from each trait across all hybrid systems. The same analysis did not reveal a significant relationship in plants ($R^2 = 0.0$, $F_{1,644} = 0.01$, $p = 0.93$).

DISCUSSION

The occurrence of phenotypic novelty through interspecific hybridization is common (Rieseberg et al. 1999) and has been suggested to be a potentially important source of adaptive genetic variation where ecological opportunity exists (Harini and Ramachandra 2003; Lexer et al. 2003b; Johnston et al. 2004; Seehausen 2004; Albertson and Kocher 2005). We predicted, based on the previous finding that transgression in hybrids is often caused by complementary gene action or epistasis (Rieseberg et al. 1999; Rieseberg et al. 2003), that the frequency of transgression should positively scale with the genetic distance between the hybridizing species. We calculated independent contrasts (Felsenstein 1985) between species pairs in genetic distance and in the proportion of transgressive traits in their hybrids to test the predicted relationship using phylogenetically controlled regressions.

Our data on 47 eudicot plant hybrid systems is consistent with our prediction. The correlation between transgression frequency and genetic distance was significantly positive. Using independent contrasts calculated between species of the same genus, more than 40% of the variance in transgression frequency was explained by genetic distance (Fig. 1a).

Inclusion of contrasts between increasingly inclusive clades caused a successive shallowing of the slope and weakening of the correlation. This could partly be an effect of increasingly different genetic architectures between lineages. The latter is supported by our finding of between-lineage variation in the frequency of transgressive phenotypes. For example, the correlation between genetic distance and transgression frequency is much stronger in rosids ($R^2 = 0.79$, $F_{1,12} = 49.96$, $p < 0.001$) than in asterids ($R^2 = 0.24$, $F_{1,16} = 5.23$, $p = 0.037$) when analysed separately on the within-genus level. An unpaired t-test (computed as the difference between the two slopes divided by the standard error of the difference between the slopes) revealed a significant difference between the slopes (unpaired *t*-tests, $t = 17.47$, p (two-tailed) < 0.001).

There is potential for measurement error in all variables we used (genetic distance, phenotypic distance and transgression frequency) deriving from a) variation in the accuracy with which phenotypic traits were reported in the literature, and b) restricting the calculation of genetic distance to only one locus (internal transcribed spacer region I and II), and c) because we assumed a clock-like evolution of this one gene. Given these possible sources of error, it is remarkable that genetic distance explains such a large proportion ($> 40\%$) of the variance in transgression frequency among the phylogenetic contrasts within genera.

Our monocot data suggests a relationship of the opposite direction such that genetically more distant species are less likely to produce transgressive hybrid phenotypes. However, none of the slopes was significant and the strength of the correlations was weaker than in the eudicot data set at all taxonomic levels. The sample size for monocots (n of different species crosses = 12) was much smaller than that for eudicots (n = 44), and it was dominated by *Allium* crosses (see Table 1). It is hence possible that the observed trend, or the absence of any strong trend, is not representative for monocot plants.

Opposite to the signal in eudicots, the animal data revealed a significantly negative correlation between transgression frequency and genetic distance (Fig. 2 a-c). However, as for monocots the taxonomic breadth of this data set was limited and dominated by one group (12 of the 15 studies were on Teleost fish, of which 8 were species crosses within the family *Cyprinidae*). Hence, we suggest handling these results with some caution. To be able to make more solid conclusions for animals, a phylogenetically more inclusive sampling is desirable. This was not possible with the data at hand.

Variation in the degree of phenotypic differentiation between parental species is a factor that needs to be taken into account when trying to asses the causes of variation in transgression frequency in interspecific hybrids. The genetic conditions allowing for complementary gene action are more likely given for traits that have been under stabilizing selection in both hybridizing species. Stabilizing selection leads to fixation of QTLs with alternating sign, that are complementary when recombined. Conversely, the probability for the appearance of transgressive hybrid offspring should be low between phenotypically divergent species. In response to divergent selection, each species is likely to have fixed alleles of same sign at multiple QTLs, but the sign being different between the species. Such genetic architecture of species differences would leave little opportunity for complementary gene action in hybrids. Transgressive phenotypes for oral jaw shape were absent amongst the hybrid offspring of two closely related Lake Malawi cichlid species with markedly different jaw morphology (Albertson and Kocher 2005). QTL sign tests implicated divergent directional selection on jaw shape in the two species (Albertson et al. 2003). Therefore, we tested if transgressive segregation frequency in interspecific hybrids was partially determined

by phenotypic differentiation of the parental species. We found our prediction strongly confirmed. In both plants and animals, the large majority of hybridizing species pairs showed a negative correlation between the extent of differentiation in a given trait, and the occurrence of transgressive expression of that trait in their hybrids (Fig 3 a, b).

The magnitude of phenotypic differentiation was not predicted by the genetic distance between species in our plant data set. It follows that in plants the predicted effect of time since speciation (genetic distance) on the occurrence of transgressive segregation was unconfounded by the potentially conflicting effects of phenotypic differentiation between species.

In contrast with plants, we found a significant positive relationship between genetic distance and phenotypic differentiation in the animal data. It is hence possible that in animals, the expected positive effect of time since speciation was masked by the expected negative effects of phenotypic differentiation. Relatively large proportions of transgressive traits observed in hybrids between closely related animal species may be a result of relatively little phenotypic differentiation, whereas distantly related species may have shown fewer than expected transgressive traits because of the relatively larger phenotypic differentiation between them.

The genetic mechanism underlying extreme trait expression can, however, not be conclusively determined from the phenotype distribution alone. If trait values are correlated with fitness, e.g. if certain traits are more strongly expressed in individuals of better constitution, transgression may also result from heterosis. Similarly, the effects of genetic incompatibilities such as Dobzhansky-Muller-interactions can lead to transgression, e.g. if reduced growth leads to smaller trait values in hybrids. Because our data are almost exclusively from first generation hybrids where heterosis is at its maximum, it is possible that increased hybrid fitness caused the expression of transgressive values in some traits. This is, however, unlikely to account for a major part of our results because at larger genetic distances the effects of heterosis on hybrid fitness are counteracted by genetic incompatibilities accumulating with time since speciation, which effectively decreases heterosis in distant crosses (Moll et al. 1965). We hence conclude that an increase in complementary gene action and epistasis are the more likely explanation for the positive relationship between genetic distance and the frequency of transgression we observed.

Since only those hybrid genotypes with heritable transgressive trait values add to the 'working surface' of natural selection, transgression based on heterosis is not expected to lead to the evolution of novel adaptations. If, on the other hand, transgression is generated by complementary gene action or epistasis, these transgressive genotypes can breed true and fixation of the most beneficial combination of parental alleles at different loci is possible (Fitzpatrick and Shaffer 2007). However, the functional relevance of the transgressive trait values detected in this analysis is mostly unknown (Lexer et al. 2003a; Gross et al. 2004; Johnston 2004) and our data make no prediction with regard to hybrid fitness. In fact some of the extreme phenotypes reported here may be mal-adaptive. Yet, under some ecological circumstances the increased working surface for selection generated by transgressive segregation in hybrids may well compensate for an average fitness loss through genetic incompatibilities (Hatfield and Schluter 1999; Via 2002), a scenario particularly relevant when novel habitats are colonized or when existing habitats have been thoroughly altered.

We conclude that both time since speciation and phenotypic differentiation have to be taken into account to predict the frequency of phenotypic novelty and the opportunity for adaptive evolution emerging from interspecific hybridization. Future work should compare transgression frequencies in hybrids from controlled crosses between closely and more distantly related species with both similar and divergent phenotypes. Such analysis should be

performed using species of a single evolutionary lineage to avoid the confounding effect of phylogenetic variance in transgression frequency.

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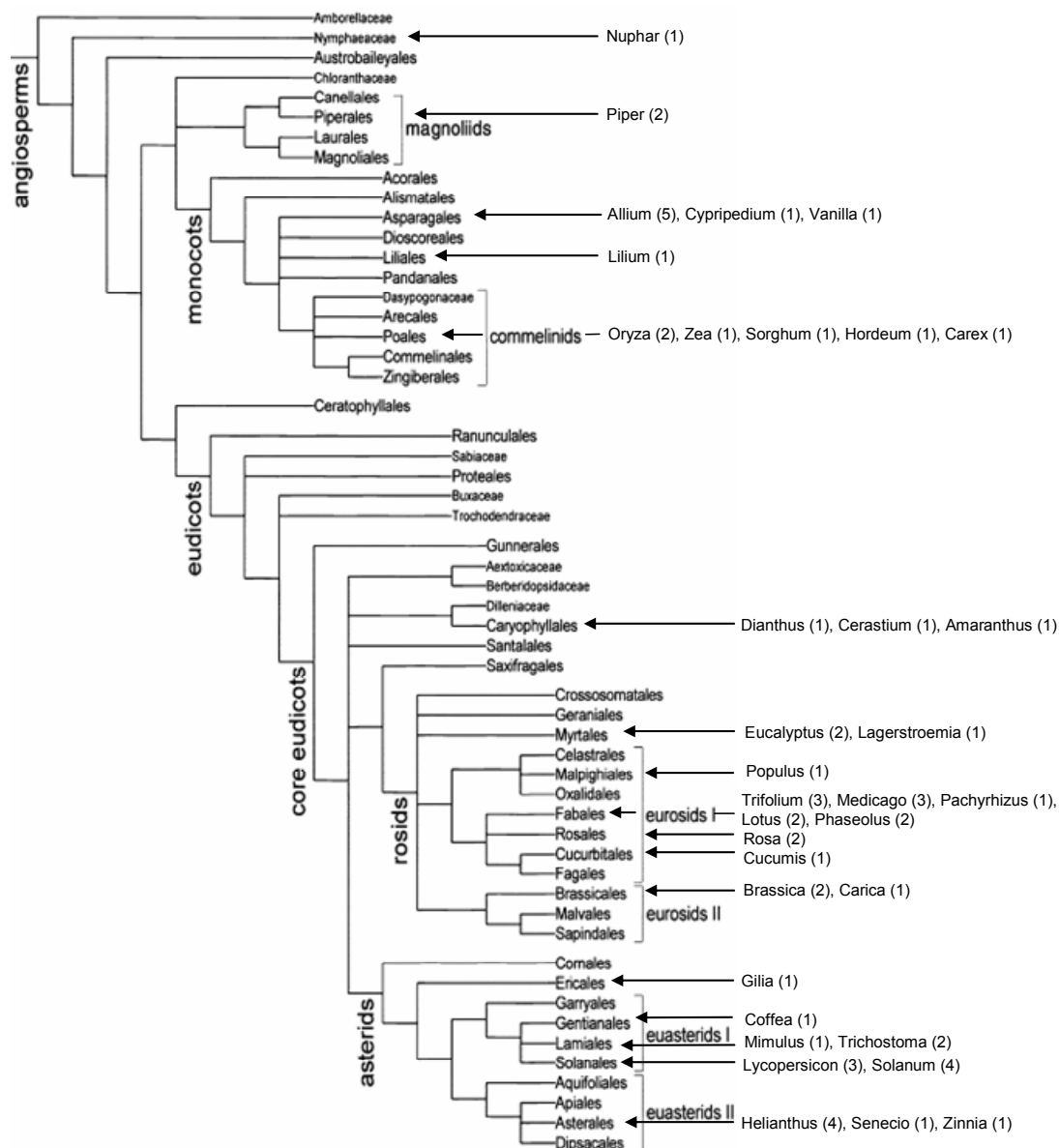
Supplementary Figure 1: Angiosperm phylogeny used for calculating independent contrasts, modified from APG II (The Angiosperm Phylogeny Group 2003). Genera included in the analysis shown on the right side of the arrows. Number of different species crosses per genus in brackets. Number of same species crosses not shown.

Protomelas taeniolatus
F1 hybrid
Astatotilapia calliptera
F2 hybrid

Rike's fish
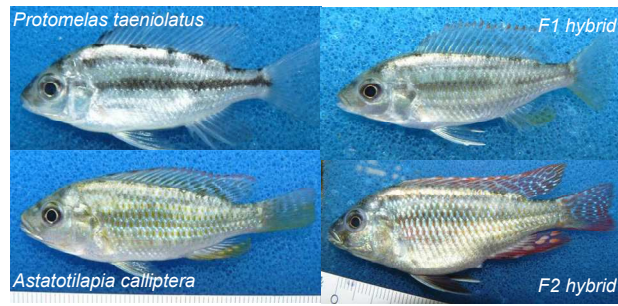
Chapter 3

The occurrence of phenotypic novelty in experimental hybrids is predicted by the genetic distance between species of cichlid fish

Rike B. Stelkens [1, 2, 3] *, Corinne Schmid [1, 2], Ole Seehausen [1, 2]

1. Division of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, Baltzerstr. 6, CH-3012 Bern, Switzerland
2. Department of Fish Ecology and Evolution, Centre of Ecology, Evolution and Biogeochemistry (CEEB), Eawag Swiss Federal Institute of Aquatic Science and Technology, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland
3. Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland

* Corresponding author: email: rike.stelkens@unil.ch; tel: +41 21 692 4249; fax: +41 21 692 4165

Experimental work and data analysis were done by RBS and CS in equal parts. RBS bred and processed F1 generation hybrids. CS bred and processed F2 generation hybrids. Assessment of growth rates of F1 hybrids and writing was done by RBS.

ABSTRACT

Transgressive segregation describes the occurrence of novel phenotypes in hybrids with extreme trait values compared to both parental species. An experimentally untested prediction is that the amount of transgression increases as the hybridizing species are increasingly less closely related. This follows from the findings of QTL studies suggesting that transgression is most commonly due to complementary gene action or epistasis which becomes more frequent at larger distances because the number of quantitative trait loci fixed for alleles with opposing signs in different species should increase with time since speciation. We measured the amount of transgression occurring in hybrids of cichlid fish bred from species pairs with gradually increasing genetic distances and varying phenotypic similarity. Transgression in multi-trait hybrid phenotypes was quantified using landmark-based geometric morphometric methods. We found that genetic distance explained 52% and 78% of the variation in transgression frequency in F1 and F2 hybrids, respectively. Transgression linearly increased with genetic distance in the F2 hybrid generation confirming our prediction. In F1 hybrids, a u-shaped relationship between transgression and genetic distance was observed suggesting that both heterosis and non-additive genetic effects caused expression of extreme trait values. Phenotypic distance between the parental species did not predict transgression in either F1 or F2 hybrids. Our data demonstrate the commonness with which novel phenotypes are produced in interspecific hybrids, and make it plausible that hybridization between divergent genomes has facilitated the unusually rapid rates of phenotypic evolution in haplochromine cichlids. This has important implications for the potential interaction of hybridization and adaptive evolution, as hybridization can generate new genotypes with adaptive potential that did not reside in either parental population, potentially enhancing a population's responsiveness to selection.

KEYWORDS: adaptive evolution, adaptive radiation, cichlids, genetic distance, hybridization, phenotypic novelty, transgressive segregation, speciation

INTRODUCTION

Hybridization as an evolutionary force has a relatively young chronicle in the literature. Despite important early work (Anderson 1949; Anderson and Stebbins 1954; Stebbins 1959; Lewontin and Birch 1966; Stebbins 1966; Templeton 1981), the image of hybridization in evolutionary literature has only recently changed from that of a merely destructive force to a more balanced view, giving due credit to hybridization's force as a potential catalyst of phenotypic diversification. Not only have cases of hybrid speciation been demonstrated conclusively both in plants and animals (Arnold et al. 1990; Lexer et al. 2003b; Schliewen and Klee 2004; Gompert et al. 2006; Mavarez et al. 2006), but hybridization is now frequently implicated in the generation of whole adaptive radiations in various groups of plants (Barrier et al. 1999; Rieseberg et al. 2003a), animals (DeSalle and Giddings 1986; Hunt et al. 1997; Feder et al. 2003; Seehausen et al. 2003; Joyce et al. 2005) and prokaryotes (Vernikos et al. 2007) (reviewed in Seehausen 2004; Mallet 2007). Besides the general surge of genetic variance ensuing from the admixture of divergent genomes (Kolbe et al. 2004), and the acquisition of specific adaptive traits through lateral gene transfer (Marri et al. 2007; Vernikos et al. 2007) and introgression (Klier et al. 1991; Parsons et al. 1993; Kim and Rieseberg 1999), another potential outcome of hybridization has been identified that may facilitate adaptive diversification into new directions: the occurrence of novel phenotypes referred to as transgressive segregation. Transgression describes the phenomenon that segregation variance in hybrid offspring can result in phenotypes with extreme trait values exceeding the range of parental trait values in either the positive or negative direction (Slatkin and Lande 1994; Rieseberg et al. 1999). Agricultural breeding programs have long benefited from transgressive phenotypes as a means to improve cultivars but studying the adaptive potential of transgression in evolutionary research is only a recent development. Transgression can in principle affect any quantitative trait and has been demonstrated for morphological traits (skull morphology of cichlid fish: Albertson and Kocher 2005), physiological traits (salt tolerance in *Helianthus* sunflowers: Lexer et al. 2003a), life history traits (flowering time in *Arabidopsis*: Clarke et al. 1995) and behavioural traits (mating behaviour of *Drosophila*: Ranganath and Aruna 2003).

Previous work on the genetic basis of transgression indicates that it is most often caused by the action of complementary genes between QTL loci that carry alleles of opposite trait signs in the parents but sum up to larger or smaller trait values compared to the parents when combined in a hybrid genome (Weller et al. 1988; DeVicente and Tanksley 1993; Mansur et al. 1993; Clarke et al. 1995; Ecke et al. 1995; Li et al. 1995; Monforte et al. 1997; Bradshaw et al. 1998; Kim and Rieseberg 1999; Rieseberg et al. 2003b). One interesting prediction emerging from this is that the amount of transgression should increase as a function of the genetic distance between the parental lines (Rieseberg et al. 1999; Seehausen 2004; Stelkens and Seehausen accepted for publication), because the number of loci for which the parents have fixed alleles with opposite effects should increase with time since isolation during the divergence of species and result more frequently in complementary gene action.

Besides genetic distance, transgression is predicted to also be affected by the phenotypic similarity of the parents (Rieseberg et al. 1999). Transgression and phenotypic differentiation have been shown to be inversely correlated such that phenotypically similar species produce more transgressive hybrid offspring than phenotypically dissimilar parents (DeVicente and Tanksley 1993; Mansur et al. 1993; Kim and Rieseberg 1999; Stelkens and Seehausen accepted for publication). This is because large phenotypic differences between two species may often result from divergent directional selection, a process expected to eventually lead to the fixation of alleles with the same trait sign across all QTL loci within a species and all opposite trait signs between the species, excluding the opportunity for complementary gene action in hybrids. Conversely, if the parents show rather similar phenotypes, despite considerably genetic distance, this indicates the action of stabilizing

selection. The genetic basis for transgression is then more likely given because stabilizing selection leads to the fixation of alleles with different trait sign at different QTLs. The trait sign fixed at every locus is random and hence likely often not the same in two species. Such alleles of opposite signs at different QTLs can then recombine to transgressive trait values in hybrids. In agreement with this prediction, a study on transgression in hybrids between two cichlid fish species revealed novel phenotypes only in traits with a selection history other than consistent directional selection (Albertson and Kocher 2005). To the extent that phenotypic and genetic divergence between species are correlated, the effects of phenotypic differentiation can potentially confound the predicted relationship between genetic distance and transgression (Rieseberg et al. 1999).

Tests on the effects of genetic and phenotypic distance on transgressive segregation remain inconclusive, mostly because the few existing studies were not designed to test this particular prediction and hence they covered only small or unknown ranges of genetic distance (Vega and Frey 1980; Cox and Frey 1985; Fabrizius et al. 1998). However, a recent comparative study (Stelkens and Seehausen accepted for publication) confirmed both; first, the predicted effect of time since speciation on transgressive segregation, finding a highly significant positive correlation in eudicot plants, but not in animals and second, the predicted negative effect of phenotypic distance on transgressive segregation which was significant in both plants and animals. Finally in animals, but not plants, phenotypic and genetic distances were highly significantly positively correlated, potentially explaining the absence of a positive effect of genetic distance on transgression.

Here, we produced seven interspecific crosses using African haplochromine cichlid fish covering a considerable range of pairwise genetic distances and varying phenotypic distances. We set out to test 1) if transgression occurred in F1 and F2 hybrids, 2) if the amount of transgression was predictable from genetic distance between the parental species, and 3) if it was predictable from the phenotypic differentiation between the parental species. To this end, we raised F1 hybrids, F2 hybrids, and non-hybrids of the two homospecific control crosses corresponding to each hybrid cross until sexual maturity under controlled laboratory conditions. To test for the presence of heterosis or genetic incompatibilities, growth rate was measured as a proxy for overall hybrid performance. Growth rates of hybrids were compared to those of homospecific crosses to test if genetic distance between the parental species had any effect on general performance. The amount of transgressive segregation per cross type was quantified using landmark-based geometric morphometric methods and a thin-plate spline procedure. Genetic distances between parental species were estimated using mitochondrial D-loop sequences from GenBank and three different molecular clocks were applied to convert distances into absolute divergence time. Multi-trait phenotypic distances between the parental species were estimated using Mahalanobis distances calculated from geometric morphometric data.

METHODS
Producing hybrids
Crosses used nine species of haplochromine cichlids from Lake Victoria and Lake Malawi (Table 2), representing different, ecologically specialized groups. Among them were a rock-dwelling planktivore (*Pundamilia nyererei*), an insect larvae picker (*Paralabidochromis chilotes*), two trophic generalists (*Pundamilia pundamilia, Paralabidochromis rockkribensis*), rock-dwelling algae scrapers (*Neochromis omnicaeruleus, Metriaclima estherae*), algae suckers (*Protomelas taeniolatus*), and two habitat generalists (*Astatotilapia calliptera, Astatotilapia burtoni*) (Seehausen 1996; Konings 2007).

All parental individuals used for making hybrid crosses were derived from laboratory populations bred from fish collected in Lake Victoria and Lake Malawi and maintained at the Eawag Center of Ecology, Evolution and Biogeochemistry Kastanienbaum, Switzerland.

Seven different F1 hybrid cross types were obtained by populating aquaria (100 x 40 x 40 cm) with five to twenty females of one species and one heterospecific male. Subsequently, F2 hybrids were bred from different males and females of six different F1 hybrid cross types (one F1 cross type, *P. rockkribensis* x *P. pundamilia*, could not be bred further due to space constraints. However, this pair was separated by the same genetic distance as two other cross types in the experiment; Table 2). No fish was used to produce more than one hybrid family.

Experimental tanks were part of a large recirculation system, light regime was 12L:12D and water temperature was kept constant at 24 - 26 °C. All animals fed a common food (dry food every day, and a blend of shrimps, peas and *Spirulina* pouder two times a week) allocated to them in equal amounts every day, and were raised to 180 days in age. At 180 days almost all individuals had reached sexual maturity. Further information regarding breeding and maintenance is given elsewhere (Stelkens et al. submitted).
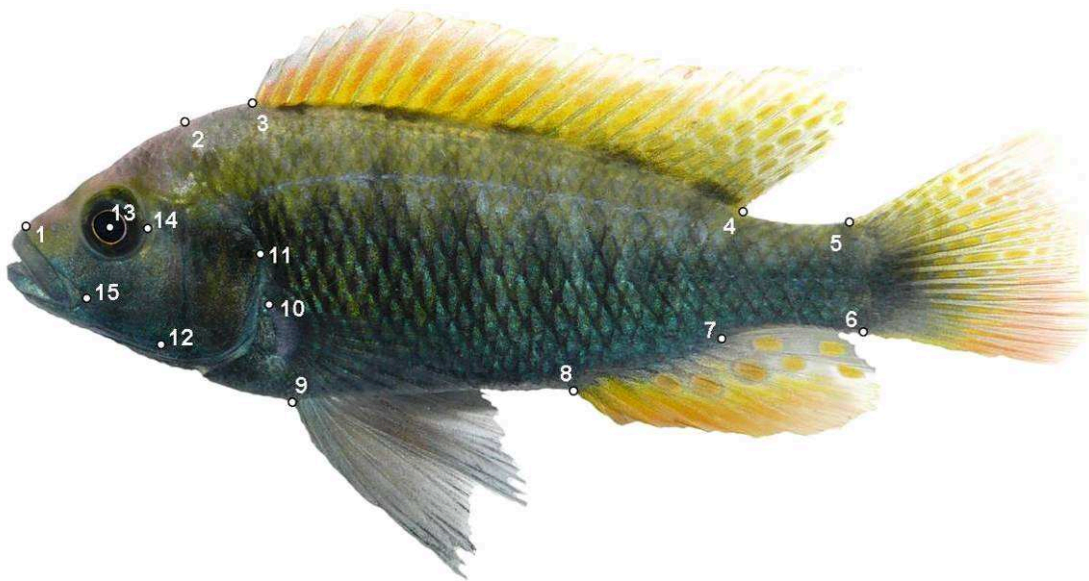

Fig. 1: F1 hybrid individual between the two African haplochromine cichlid species *Pundamilia nyererei* and *Astatotilapia calliptera*. Numbers label the 15 landmarks used for geometric morphometric analysis of body shape variation in interspecific hybrids. 1) Anterior tip of maxilla, 2) junction of head and dorsal scales, 3) anterior insertion of dorsal fin, 4) posterior insertion of dorsal fin, 5) dorsal junction of caudal fin and caudal peduncle, 6) ventral junction of caudal fin and caudal peduncle, 7) posterior insertion of anal fin, 8) anterior insertion of anal fin, 9) anterior insertion of pelvic fin, 10) dorsal insertion of pectoral fin, 11) posterior reach of operculum, 12) lower margin of preopercule, 13) centre of the eye, 14) anterior insertion of the preopercule, and 15) anterior reach of the premaxillary groove.

Measuring transgressive segregation using geometric morphometrics
All hybrids and the corresponding homospecific individuals were photographed at the age of 180 (± 1) days. Pictures were taken of the left side of the live fish in a transparent photo cuvette with a scale for size calibration. Geometric morphometric analysis was performed on the x-y coordinates of 15 landmarks placed on the photographs (Fig. 1) using tpsDig version 2.10, (Rohlf 2006). To reduce noise introduced through variation in position, orientation and size, this non-shape variation was mathematically removed using generalized procrustes analysis (GPA) (Gower 1975; Rohlf and Slice 1990). GPA superimposes landmark configurations in that it minimizes the sum of squared distances between corresponding landmarks by scaling, translating and rotating specimens onto a mean consensus configuration calculated from all specimens. Thin-plate spline (TPS) procedure was then applied to obtain

partial warps using tpsRelw version 1.45 (Rohlf 2007). Partial warps estimate the minimum bending energy needed to deform an infinitely thin metal plate (i.e. the landmark configuration of an individual fish) to adopt the shape of another landmark configuration (i.e. the consensus configuration among all the fish) while being constrained at particular points (i.e the landmarks). The total deformation of the spline can be broken down into geometrically orthogonal components in a Cartesian coordinate system (i.e. the partial warps) to describe the amount of stretching, bending and twisting necessary to superimpose the coordinates of all specimens onto the consensus shape. Each individual then has a weight for the x- and y- components of each partial warp.

All subsequent analyses were performed in JMP 7.0 (SAS 2006). Partial warp weights were regressed against size and residuals of these were used for all further analysis to remove potential allometric size effects. Residuals were entered into principal component analysis (PCA) to identify the major axes of shape variation, which is also referred to as relative warp analysis (Bookstein 1996). We extracted all principal components that explained more than 5% of the variance in the data set (between 4 and 6 components across all crosses).
The amount of transgression (T_{PCi}) occurring along a principal component axis (PC_i) was calculated as

equation (1)
$$T_{PCi} = \frac{\left(range_{total} - range_{homospecific}\right)}{range_{homospecific}},$$

where $range_{total}$ is the total phenotypic range between the largest and smallest observation of all hybrid and homospecific individuals of a particular cross type, and $range_{homospecific}$ represents the phenotypic range including only homospecific individuals of that cross type. The numerator hence stands for the transgressive portion of the hybrid range ($range_{trans}$; for a schematic drawing of the variables used see Fig. 2). We then calculated the sum of transgression found along all PCs to obtain the total amount of transgression (T_{total} which can be larger than 100%). This was done in a weighted averaging procedure, where T_{PCi} was multiplied with the percentage variance explained by that PC.

To test the effect of increasing genetic distance on the amount of transgression (T_{total}) we regressed T_{total} against genetic distance using linear regression models. Normal distribution of variables was confirmed with Shapiro-Wilk tests.

To test whether families within cross types differed in phenotype, we used ANOVA with family as factor and all relevant PCs as response variables. This analysis was performed on both hybrids and homospecific crosses.
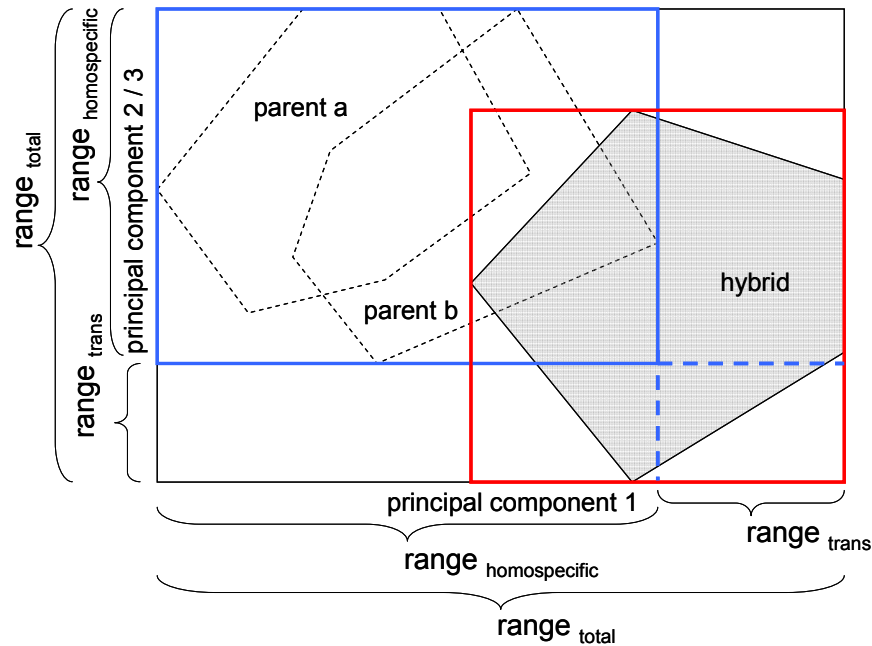
Fig. 2: Schematic drawing of the variables used in equation 1 (see Methods) to quantify the amount of transgression found in hybrid crosses.

Measuring genetic distance and divergence time

Genetic distances were estimated for every species pair used for making hybrid crosses by calculating uncorrected p-distances from D-loop sequences downloaded from NCBI GenBank (http://ncbi.nlm.nih.gov/Genbank/; accession numbers of all sequences can be found in Supplementary Table 1). All available sequences of every species were included for calculating genetic distances. For five species no sequences were available. In these cases we used sequences from a very closely related species. This was in all cases justified because both species (experimental and substitute) belonged to the same clade within which mitochondrial DNA lineage sorting is highly incomplete (i.e. the radiation of Lake Victoria and a clade of the Lake Malawi Mbuna). Sequences were aligned in ClustalW (Thompson et al. 1997) using the pairwise alignment algorithm and alignments were manually controlled and improved locally. Genetic distances were calculated in MEGA 4 (Kumar et al. 2004). Where multiple sequences were available, we took the average of all possible pairwise interspecific p-distances (e.g. Mendelson 2003; Chapman and Burke 2007). To correct comparisons between species for the variation occurring within species, mean intraspecific genetic distances (the mean of the two species means) were subtracted from mean interspecific distances (Nei 1987; Mendelson 2003).

Table 1: Hypothetical example of segregation variance involving three-locus, two-allele diploid genotypes in hybrids between populations (or species) 1 and 2. Small letters decrease the trait value by one unit; capital letters increase the trait value by one unit. Not all possible hybrid genotypes are shown, only those with maximal and minimal (transgressive) trait values. Net trait scores are shown in brackets.

genetic effect	parental population 1	parental population 2	F1 hybrids	F2 hybrids
additive	AAbbCC (+1)	aaBBcc (-1)	AabBCc (0)	AABBCC (+3), aabbcc (-3)
dominant	AAbbCC (+1)	aaBBcc (-1)	AabBCc (+3)	AABBCC (+3), aabbcc (-3)

Genetic distances were converted into absolute times of divergence using two different non-linear relaxed molecular clocks, one calibrated using the cichlid fossil record and recent geological events and the other using the fragmentation of Gondwanaland and recent geological events (Genner et al. 2007). Additionally, we used an internally calibrated linear clock that has been widely used in cichlid phylogeography (Sturmbauer et al. 2001).

Measuring phenotypic divergence

Phenotypic divergence was quantified by measuring the mean of all Mahalanobis distance between any two species. As variables we used all principal components (from a PCA including all parental species) that explained more than 5% of the variance. Distances were then averaged to obtain a measure of the overall phenotypic dissimilarity of any two parental species. To correct comparisons between species for the variation occurring within species, mean intraspecific phenotypic distances (the mean of the two species means) were subtracted from mean interspecific distances.

To test the effect of increasing phenotypic distance on the amount of transgression, T_{total} was regressed against phenotypic distance using linear regression models. Normality of distribution was confirmed with Shapiro-Wilk tests.

Table 2: Seven different interspecific hybrid crosses and nine homospecific crosses used to measure transgressive segregation with the number of families per cross type. The number of individuals per family, that were photographed and measured, is shown in brackets. Sex-reversed crosses of the same cross type are indicated by 'a' and 'b'.

cross type	hybrid crosses		n families (n individuals)	
	male parent	**female parent**	**F1 hybrids**	**F2 hybrids**
1	*Neochromis omnicaeruleus*	*Pundamilia pundamilia*	4 (21,29,45,33)	4 (5,10,19,9)
2	*Paralobidochromis chilotes*	*Pundamilia nyererei*	2 (24,19)	5 (19,21,2,3,8)
3	*Paralobidochromis rockkribensis*	*Pundamilia pundamilia*	3 (37,26,43)	-
4°	*Astatotilapia calliptera*	*Metriaclima estherae*	5 (3,11,11,9,6)	8 (2,22,16, 4, 6, 7,4,7)
4b	*Metriaclima estherae*	*Astatotilapia calliptera*	3 (2,21,16)	4 (4,12,11,10)
5	*Protomelas taeniolatus*	*Astatotilapia calliptera*	2 (21,43)	7 (12,12,13,4,14,5,12)
6	*Astatotilapia burtoni*	*Astatotilapia calliptera*	4 (6,2,19,19)	5 (9,10,15,17,16)
7	*Pundamilia nyererei*	*Astatotilapia. calliptera*	8 (15,20,5,15,20,30,22,28)	8 (4,18,4,1,9,4,6,17)

cross type	homospecific crosses	n families (n individuals)
1	*Pundamilia nyererei (P. ny)*	3 (33, 35, 8)
2	*Pundamilia pundamilia (P. pun)*	3 (27, 15, 16)
3	*Neochromis omnicaeruleus (N. omni)*	3 (7, 5, 30)
4	*Paralobidochromis rockkribensis (P. rock)*	3 (18, 27, 18)
5	*Paralobidochromis chilotes (P. chil)*	3 (17, 4, 16)
6	*Metriaclima estherae (M. est)*	3 (29, 23, 5)
7	*Astatotilapia burtoni (A. burt)*	3 (11, 16, 16)
8	*Astatotilapia calliptera (A. call)*	3 (38, 48, 27)
9	*Protomelas taeniolatus (P. taen)*	3 (9, 26, 22)

Measuring growth rate

Each individual was photographed at six age brackets (day 30, 60, 90, 120, 150, 180 ± 1 day) with a digital camera (Nikon Coolpix 8700). For this, the camera was fixed on a tripod vertically above a transparent Petri dish containing water and a linear scale. The scale was used during picture analysis to correct fry size for variation in camera distance. The Petri dish

was illuminated from below to obtain contrast-rich pictures. Fry were put into the dish in small numbers (typically 5 to 10, depending on their body size). Picture analysis to measure fry size was performed in ImageJ (http.//rsb.info.nih.gov/ij/download.html) by changing the coloured photos into black-and-white pictures. Each black section in the picture was converted into area coverage (cm^2) indicative of the individual's body size (van der Sluijs et al. 2008). The measurements were used to calculate growth rates during the first 180 days. Mean growth rates were calculated by averaging over all individuals within a cross type.

Hybrid growth was then compared to the growth of both corresponding homospecific crosses by regressing body size on time in linear regressions forced through the origin. Slopes of regressions were compared using paired Student's t-tests assuming equal variances and computed as the difference between the two slopes divided by the standard error of the difference between the slopes using R (R 2006). Growth was compared among hybrid crosses to test if hybrid growth rates depended on the genetic distance between the parental species. As above, slopes were compared using paired Student's t-tests. Finally, we calculated the differential between hybrid and mean parental growth per cross type and regressed the differential against genetic distance.

RESULTS

Transgressive segregation in hybrids

Thirty F1 hybrid families from seven different cross types and three families of each corresponding homospecific cross were obtained (see Table 2 for number of families and number of individuals per hybrid and homospecific cross type). Transgressive phenotypes were found in all hybrid cross types (Table 3; Supplementary Fig. 1). F1 hybrids exceeded the phenotypic range of the parental species by 14% $\pm$ 13% (averaged across all cross types and across all axes of shape variation, weighted by the % variance each axis explained).

Forty-one F2 hybrid families from six different cross types were obtained (Table 2). Transgression was observed in all cross types (Table 3; Fig 3). F2 hybrids exceeded the phenotypic range of the parental species by 21% $\pm$ 12% (averaged across all cross types and across all axes of shape variation, weighted by the % variance each axis explained). The amount of transgression and variance explained by each PC axis for both F1 and F2 hybrids is shown in Supplementary Table 2.

Within each cross types, there were significant differences between families in the distribution of phenotypes in morphospace in both F1 and F2 hybrids. ANOVAs with family as factor and all relevant PC axes as response variables suggested that within cross type, at least one hybrid family was significantly different from another family along at least one axis of shape variation (all test results shown in Supplementary Table 3). However, transgression analysis revealed that on average 75% (2-5 families) of all F1 families and 84% (3-7 families) of all F2 families contained transgressive phenotypes demonstrating that transgressive segregation was not caused by single-family effects (Table 3).
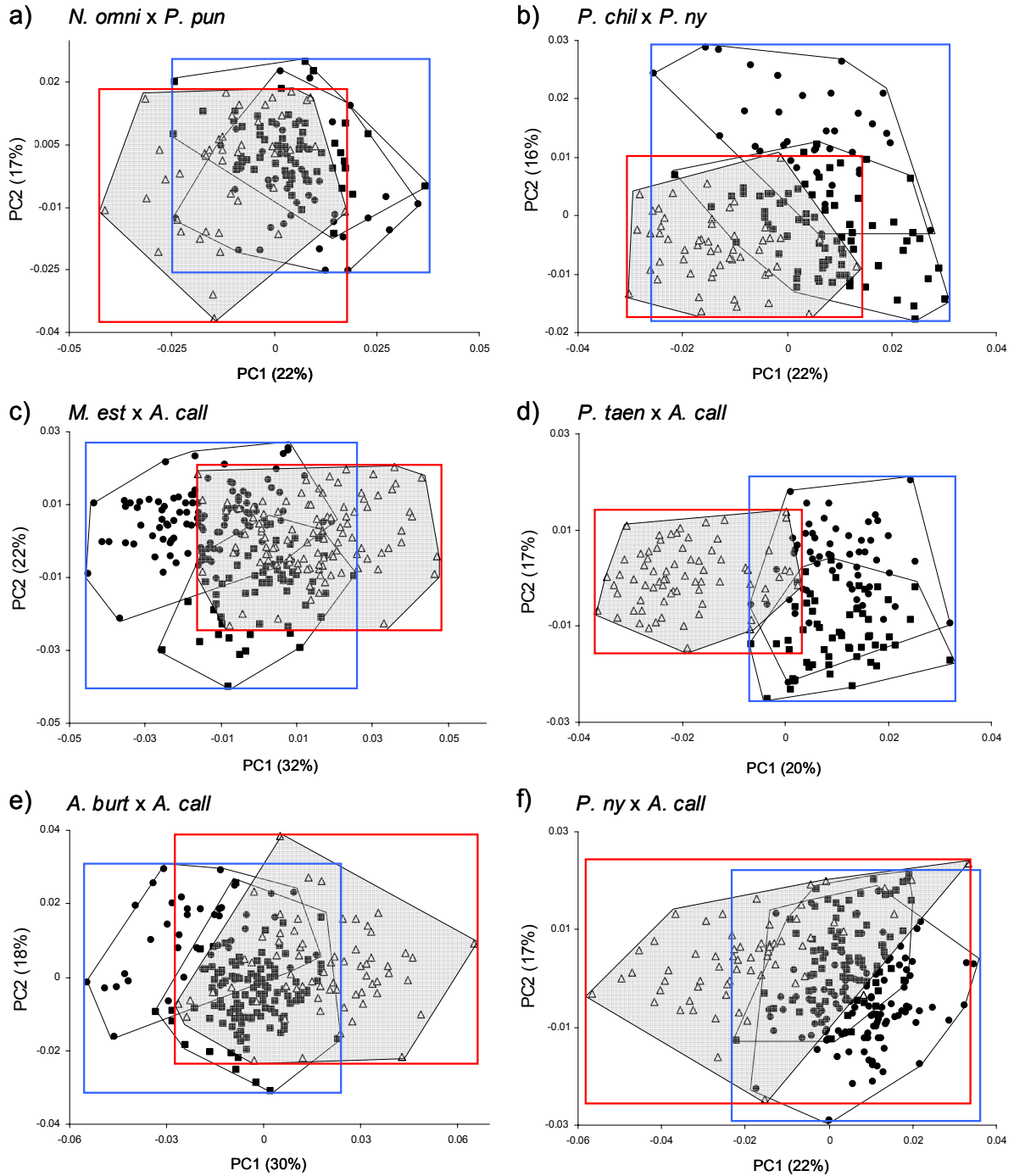
Fig. 3: Results of principal component analyses using geometric morphometrics data to quantify the amount of transgression in shape of interspecific hybrids of haplochromine cichlids. Graphs show the distribution in morphospace of six different F2 hybrid crosses and the corresponding homospecific crosses of species pairs with increasing genetic distance from smallest (a, b) to largest distance (f). Abbreviations of species names correspond to Table 2. Every data point represents one individual. Filled symbols indicate parental species, triangles indicate F2 hybrids. Blue squares encompass the phenotype range of the combined parental species; red squares represent the phenotype range of F2 hybrids. The percentage of variance explained by principal component 1 and 2 are shown in brackets. Note that the visualization of transgression is restricted to the first two axes of shape variation here, which is not (or not entirely) representative of the total amount of transgression found per cross type.

Table 3: All hybrid crosses with pairwise genetic distances (uncorrected p-distance calculated from mitochondrial D-loop sequences), divergence times (in millions of years based on two different relaxed molecular clocks and the lower-upper bounds of the internally calibrated "Sturmbauer clock") and phenotypic differentiation based on Mahalanobis distances. The total amount of transgression (T_{total}) occurring on the major axes of phenotypic shape variation is shown separately for F1 and F2 hybrids.

cross type	species crossed	genetic distance	divergence time internal clock	divergence time fossil record	divergence time Gondwana break up	phenotypic distance	% T_{total}	transgressive families (%)	% T_{total}	transgressive families (%)
							F1 hybrids		F2 hybrids	
1	*N. omni x P. pun*	0.007	0.35-0.61	0.104	0.135	5.69	12.55	75	14.73	100
2	*P. chil x P. ny*	0.007	0.35-0.61	0.104	0.135	3.01	30.76	100	6.42	80
3	*P. rock x P. pun*	0.007	0.35-0.61	0.104	0.135	6.18	32.48	100	-	-
4	*M. est x A. call*	0.0188	0.93-1.64	0.58	0.919	23.98	0.14	37.5	18.20	71.4
5	*P. taen x A. call*	0.0241	1.19-2.1	0.891	1.485	11.62	3.71	100	14.40	100
6	*A. burt x A. call*	0.0408	2.02-3.56	2.226	4.117	15.29	5.58	50	39.07	100
7	*P. ny x A. call*	0.0553	2.74-4.82	3.779	7.426	14.97	13.9	62.5	32.46	75

Transgressive segregation as a function of genetic distance

Uncorrected pairwise p-distances between the species pairs, calculated from D-loop sequences, ranged from 0.007 to 0.0553. Depending on the molecular clock used, this translates into a range of absolute time since speciation from several thousand years to 2.7/3.8/7.4 million years (Table 2; lower time estimate of the internal clock/fossil record calibration/Gondwana fragmentation calibration; from here on results of the three different molecular clocks will be reported in this order).

In F1 hybrids, testing genetic distance as a predictor for the total amount of transgression (T_{total}) resulted in a u-shaped relationship (quadratic regression: R^2 (adjusted) = 0.52, $F_{2,6}$ = 4.21, p = 0.104; Fig. 4a). Large amounts of transgression were observed in hybrids between both closely (13-33%) and distantly related crosses (14%) with a near absence of transgression in crosses of intermediate genetic distance (0.1-6%).

In F2 hybrids, transgression significantly increased with genetic distance (linear regression: R^2 = 0.78, $F_{1,5}$ =12.29, p = 0.025; Fig. 4b) with a minimum of 6% transgression in closely related crosses and a maximum of 39% transgression in distant crosses.

Fig. 4: Total amount of transgression (T_{total}) observed in interspecific a) F1 hybrids and b) F2 hybrids as a function of genetic distance (uncorrected p-distance) between the parental species. Regression lines are from quadratic (a) and linear (b) model fitting. The solid line indicates a (significant) linear relationship in F2 hybrids, the dotted line indicates a (nonsignificant) quadratic relationship in F1 hybrids.
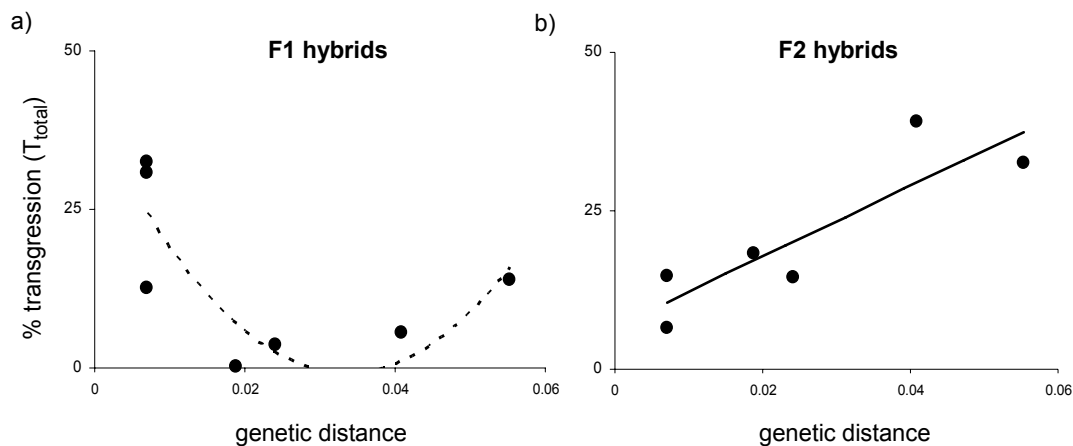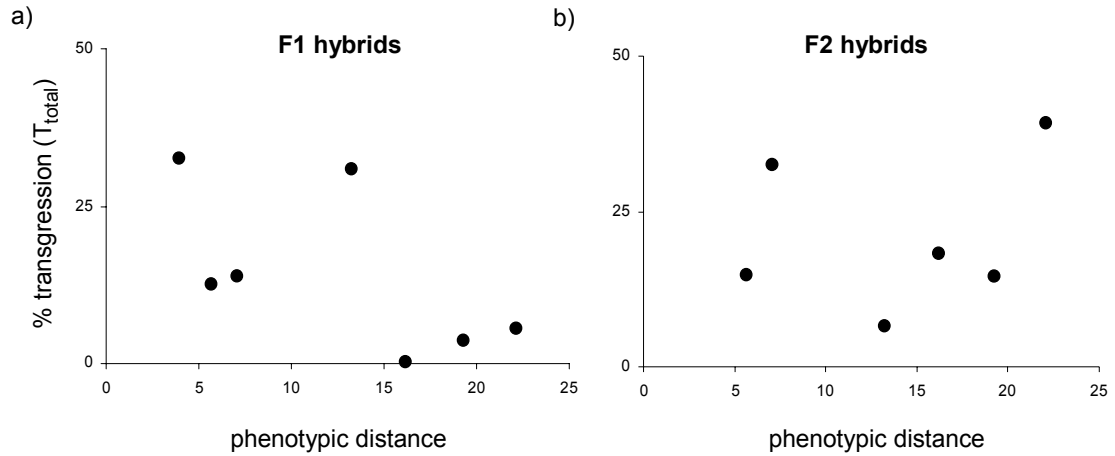
Fig. 5: Total amount of transgression (T_{total}) observed in interspecific a) F1 hybrids and b) F2 hybrids as a function of the phenotypic distance (Mahalanobis distance calculated from 15 geometric morphometric landmarks) between the parental species. No significant relationships were found.

Transgressive segregation as a function of phenotypic differentiation

According to our prediction, transgression should decrease as a function of phenotypic dissimilarity between the parental species. Testing phenotypic distance (calculated a Mahalanobis distances) as a predictor for the amount of transgression (T_{total}) did not result in a significant relationship in F1 hybrids (linear regression: $R^2 = 0.38$, $F_{1,6} = 3.06$, $p = 0.140$; Fig. 5a) or in F2 hybrids (linear regression: $R^2 = 0.05$, $F_{1,5} = 0.21$, $p = 0.674$; Fig. 5b).

We further tested if phenotypic and genetic divergence between the parental species were correlated. Although we found a positive trend, the relationship was not significant (logarithmic regression: $R^2 = 0.22$, $F_{1,6} = 1.45$; $p = 0.28$; Fig. 6) due to one outlying data point (*A. calliptera x M. estherae*, cross 4; exclusion of this outlier resulted in a strong positive correlation: $R^2 = 0.81$, $F_{1,5} = 17.32$; $p = 0.014$).
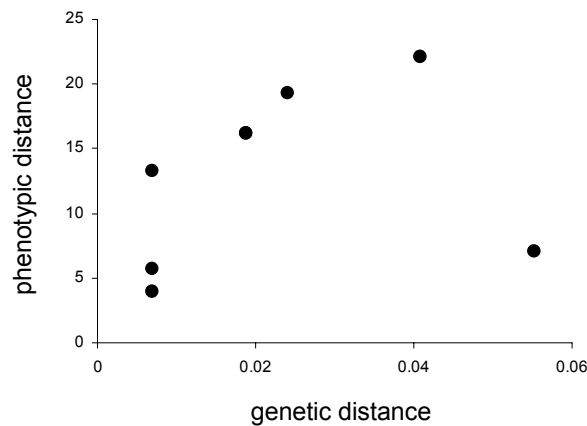

Fig. 6: Phenotypic divergence (Mahalanobis distance calculated from 15 geometric morphometric landmarks) of parental species   as a function of the genetic distance (uncorrected p-distance) between them. No significant relationship was found due to one outlying data point.

The effect of genetic distance on hybrid growth

When parental species had different growth rates, represented by the slope of the regression of body size on time (four cases), hybrid growth was either intermediate between growth of the two corresponding homospecific crosses (three cases), or resembled that of one parental species (1 case; Fig. 7). When parental species had similar growth rates, hybrid growth was either intermediate but not significantly different from the parents (Fig. 7 e, f), or in one case, the cross between *A. calliptera* and *M. estherae* (Fig. 7d), hybrids grew significantly faster and reached larger size than both parental species.

cross	gen D	hybrid axb vs. bxa	hybrid vs. parent a	hybrid vs. parent b	parent a vs. parent b
a	0.007	-	p < 0.001	p < 0.001	p < 0.001
a	0.007	-	p < 0.001	p < 0.001	p < 0.001
c	0.007	-	p < 0.001	p = 0.562	p < 0.001
d	0.0188	p < 0.001	p (axb) = 0.139 p (bxa) < 0.001	p (axb) < 0.001 p (bxa) < 0.001	p = 0.03
e	0.0241	-	p = 0.081	p = 0.372	p = 0.974
f	0.0408	-	p = 0.131	p = 0.605	p = 0.55
g	0.0553	-	p < 0.001	p = 0.004	p < 0.001
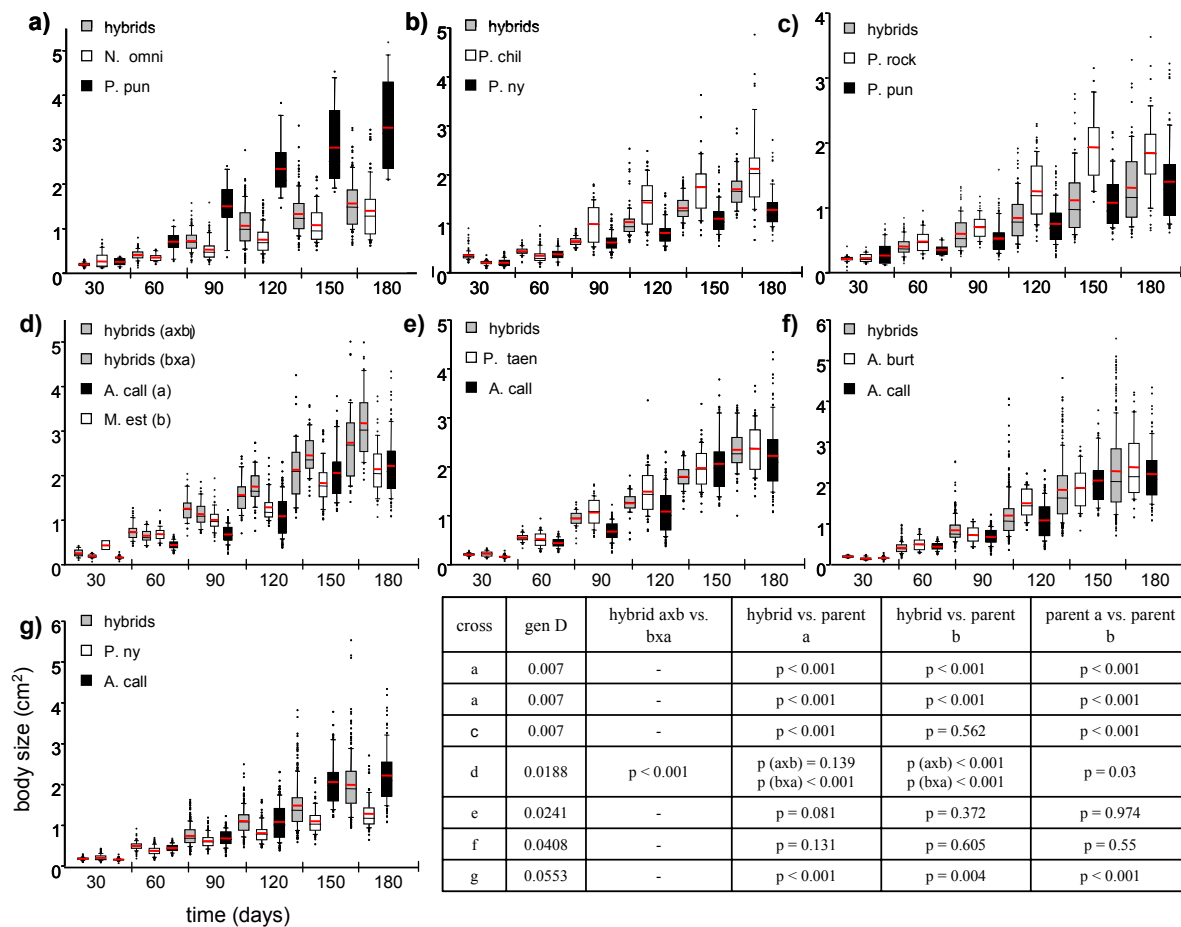
Fig 7: Growth rate measured as body size from the age of day 30 to day 180. Panels (a-g) show the seven different interspecific F1 hybrid crosses and their corresponding homospecific crosses. Lower and upper box hinges show 25th and 75th percentiles, black line shows median, red line shows mean. Whiskers indicate minimum and maximum observations. Data points outside the boxes represent outliers. Panel d) shows the only case where reciprocal crosses were obtained. The inserted table shows results of pairwise Student's t-tests comparing the slopes of the hybrid and the corresponding homospecific regression lines. Abbreviations of species names correspond to Table 2. Number of individuals per family and number of families per cross type can be found in Table 2.

In hybrids, growth rate (% gain in body size over time) significantly decreased over time in logarithmic regression models (all cross types: $R^2 > 0.73$, $p < 0.05$; except for *P. chilotes* x *P. nyererei*: $R^2 = 0.0$; $F_{1,4} = 0.0$, $p = 0.97$). Comparing the slopes of hybrid growth rates revealed that only *N. omnicaeruleus* x *P. pundamilia* and *P. rockkribensis* x *P. pundamilia* differed

significantly from one another in that the gain of body size decreased more slowly in the latter (t = 3.76, p = 0.006). All remaining cross types had similar slopes, suggesting that the genetic distance between species had no impeding or accelerating effect on the growth rates of their hybrids.

Testing the growth slope differential (hybrid growth - mean parental growth) against genetic crossing distance revealed that hybrid growth was not affected by genetic distance (R^2 = 0.08; $F_{1,7}$ = 0.51, p = 0.50).

DISCUSSION

Numerous studies on interspecific animal and plant hybrids have demonstrated that hybridization frequently gives rise to phenotypic novelty. One source of such novelty that may facilitate adaptive evolution, is transgressive segregation which refers to the occurrence of hybrid phenotypes that express trait values exceeding the phenotypic range of both parental species combined with either a positive or negative sign (Slatkin and Lande 1994; Rieseberg et al. 1999). Evidence supporting the notion that transgressive ecomorphological and ecophysiological trait values can be beneficial and permit colonizing previously underutilized peaks on a fitness landscape, comes from detailed work on *Helianthus* sunflowers. This work conclusively demonstrated how transgression in key ecological traits can allow hybrids to escape the homogenizing effects of gene flow from parental species through invading an ecologically and spatially distinct niche (Schwarzbach et al. 2001; Lexer et al. 2003b; Rieseberg et al. 2003a; Gross et al. 2004).

Quantitative genetics work has consistently found evidence for complementary gene action to be the most frequent cause of transgressive segregation (reviewed in Rieseberg et al. 2003b) although epistatic interactions (Monforte et al. 1997) and non-additive effects by overdominance within loci (DeVicente and Tanksley 1993) can also contribute to a lesser extent. Despite this knowledge of the genetic basis of transgression, so far, only one study has formally tested the relationship between genetic distance and transgression (Stelkens and Seehausen 2009), and the prediction that distantly related species more often produce hybrids with extreme trait values than closely related species (Rieseberg et al. 1999). This is expected because the number of quantitative trait loci fixed for alleles with opposing sign in different species should increase with time since speciation, leading to more frequent opportunity for complementary gene action and epistasis.

Here, we used African haplochromine cichlid fish from two large adaptive radiations (Lake Victoria, Lake Malawi) to test if the amount of transgression in interspecific hybrids increased as a function of genetic distance between species. We made seven different cross types from different species combinations representing five different genetic distances, covering absolute divergence times of between a few thousand years to 2.7/3.8/7.4 million years depending on the molecular clock used (lower time estimate of the internal clock/fossil record calibration/Gondwana fragmentation calibration; Table 2). Using geometric morphometrics on the multi-trait phenotypes we quantified and compared shape variation in F1 and F2 generation hybrids and in the two corresponding homospecific control crosses.

We found that transgressive segregation was frequent and extreme trait values were observed in each F1 and F2 cross generated. Further, the amount of transgression observed in F2 hybrids increased linearly with time since speciation (Fig. 4b) confirming our prediction. Interestingly, in the F1 generation large amounts of transgression were expressed in hybrids between both closely and distantly related species but transgression was nearly absent in hybrids of parents with intermediate genetic distances, resulting in a u-shaped relationship between transgression and divergence time (Fig. 4a). While the increase of transgression in F1 hybrids of distant crosses can be explained by a higher frequency of epistatic interactions and dominant genetic effects prevailing at some of the loci contributing to complementary

gene action (Table 1), the large amount of transgression observed in F1 hybrids of closely related species was unexpected.

If we were to speculate, it is possible that an overall increased fitness, accompanying increased average heterozygosity in the F1 hybrid generation, may have led to larger, and hence often transgressive trait values in more healthy individuals of crosses between closely related species. Generally, the relationship between offspring fitness and the genetic distance between parents is predicted to be hump-shaped (with a left-shifted mode) confirmed by both experimental (Moll et al. 1965; Waser 1993; Marshall and Spalton 2000; Waser et al. 2000; Neff 2004; Willi and Van Buskirk 2005) and theoretical work (Campbell and Waser 1987; Schierup and Christiansen 1996). Due to inbreeding depression (typically observed in intraspecific, within-population crosses, (Charlesworth and Charlesworth 1987)) offspring fitness should initially increase with genetic distance between parents. Then, at larger genetic distances, heterosis effects would no longer come to bear as they are increasingly concealed by the counteracting effects of genetic incompatibilities, the break-up of co-adapted gene complexes, epistatic interactions and underdominance (heterozygote disadvantage), which can all decrease the fitness of distant crosses (Lynch 1991; Edmands 2002). The latter defines the region of outbreeding depression, generally between distantly related conspecifics and heterospecifics. Hence, the fitness peak will typically reside in the area of intraspecific between-population crosses. However, when speciation was very recent, as in rapidly radiated species flocks, the intrinsically determined fitness peak (i.e. excluding extrinsically ecologically determined fitness) may well be shifted into the interspecific area.

It would be useful to determine the genetic distance where the increasing effects of genetic incompatibilities and heterosis effects typically cancel out, to assess if this may have caused the depression in the amount of transgression at intermediate distances in F1 hybrids observed in our experiment. However, comparing the results of the above literature (Moll et al. 1965; Waser 1993; Marshall and Spalton 2000; Waser et al. 2000; Neff 2004; Willi and Van Buskirk 2005) proved impossible due to variation in methods used to estimate genetic distance (geographic distance, sequence divergence of different genes, F_{st}, allozyme variation) and the large array of different mating systems investigated. We hence conducted a literature search on Web of Science (http://portal.isiknowledge.com/portal.cgi) using the keyword combinations (i) optimal outbreeding (31 hits), (ii) fitness AND genetic distance AND hybrid* (48 hits), (iii) heterosis AND outbreeding depression (113 hits), and (iv) heterosis AND genetic incompatib* (350 hits). While some studies confirmed a decrease of fitness in F1 hybrids at large outcrossing distances (e.g. Moll et al. 1965; Xiao et al. 1996; Willi and Van Buskirk 2005), others observed no effect (e.g. Edmands 1999; Stokes et al. 2007). Again the inconsistency across studies in genetic markers and other methods used to determine genetic distance did not allow us to determine the divergence time after which genetic incompatibilities and heterosis effects typically cancel out.

In our own experiment, hybrid fitness, estimated from growth rates, showed no evidence for either effects of heterosis or genetic incompatibilities in the F1 generation (Fig. 7). Hybrid growth rates, regardless of the genetic distance between species, were mostly intermediate between the two corresponding homospecific control crosses, and were not affected by the genetic distance between parents. Only in one cross (cross 4, *A. calliptera* axnd *M. estherae*; Fig. 7d) did the hybrids grow faster than the parental species. Surprisingly, this was the cross with the lowest amount of transgression observed in F1 hybrids (Fig. 4a). This finding does not support the hypothesis that overall increased heterozygosity of hybrid genotypes caused the transgression observed in our experiment. Conversely, the data also support that the observed transgression was not caused by developmental instabilities due to genetic incompatibilities.

We found, that the degree of phenotypic differentiation of the parental species in our experiment was not strongly predicted by genetic distance (Fig. 6). Because phenotypic

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distance is, in contrast to genetic distance, predicted to have a negative effect on the occurrence of transgression, the effects of both variables can theoretically cancel each other out. We thus tested if transgression was also a function of the increasing phenotypic dissimilarity between species. Contrary to our prediction, the amount of transgression in both F1 and F2 hybrids was independent of phenotypic differentiation (although a slight negative trend was present in the F1 generation; Fig. 5a). We can hence exclude the possibility that the effect of genetic distance was compromised by the counteracting effects of phenotypic divergence in our experiment.

Except for the three species crosses representing the lowest end of the genetic distance gradient in our experiment, most of the species we used are allopatric in the wild (crosses 4-7, Table 3). They presumably acquired divergent phenotypes as a result of drift and different selection regimes in different environments rather than due to consistent and strong disruptive selection on the same traits, which would have purged many of the antagonistic allelic effects within QTLs. It is hence likely that alleles of opposing signs were preserved during the divergence of even the phenotypically most divergent species in our experiment, resulting in frequent opportunity for complementary gene action in their hybrids. The latter may explain why the amount of transgression is not a function of phenotypic divergence in our data set. Our experimental design is not suitable to test the effect of a gradually increasing disruptive selection coefficient on the amount of transgression but this relationship is certainly worthwhile to be investigated in future experiments.

All factors considered it seems plausible that the observed increase in transgression with genetic distance in F2 hybrids is mainly the result of an increasing opportunity for complementary gene action and epistasis given in hybrids between more divergent lineages. This is due to an increasing number of loci for which the diverging species fix alleles with opposite signs (excluding the case of consistent selection into different directions, where one species becomes fixed for only positive signs and the other becomes fixed for only negative signs across all QTLs within a species), providing more frequent opportunity for transgression.

Implications of the observed positive relationship between genetic distance and transgression are particularly interesting where hybridization between distantly related lineages has taken place at the onset of adaptive radiations. Traces of ancient hybridization in phylogenetic reconstructions of several plants and animal radiations suggest that genetic exchange between at least two distantly related lineages occurred at the onset of radiations, and may have acted as a catalyst for the phenotypic diversification of these groups (Seehausen 2004; Joyce et al. 2005; Day et al. 2007; Vernikos et al. 2007; Friar et al. 2008). The largest genetic distance between species in our experiment represents similar divergence times (2.7/3.8/7.4 my) to those estimated for the hypothesized multiple ancestors of two major cichlid radiations (Lake paleo-Makgadikgadi (Joyce et al. 2005), Lake Victoria (Seehausen et al. 2003).

Here we showed that most phenotypic novelty occurs when genetically distant species hybridize, and that hybridization between distant species of East African cichlids indeed generates very significant amounts of phenotypic novelty. It is worth mentioning that many of the hybrids we obtained resemble in morphology and colour other species known from the cichlid radiations. These results make it indeed plausible that hybridization between divergent genomes has facilitated the unusually rapid rates of phenotypic evolution in haplochromine cichlids. The innate capacity of transgressive segregation to massively increase the working surface for selection within just two generations, theoretically allows phenotypic diversification of a group with new momentum without the long waiting time for new mutations. Some hybrid species have indeed been shown to establish in completely new ecological niches in very few generations (Lexer et al. 2003a; Nolte et al. 2005). If transgressive segregation was indeed an important contributor to the volume and extent of

phenotypic diversification during adaptive radiations (Albertson et al. 2003; Seehausen 2004; Albertson and Kocher 2005), variation in the genetic architecture between lineages (which can be either conducive or obstructive to complementary gene action) might cause variation in the rates of adaptive radiation observed between lineages. This hypothesis is speculative at this moment and awaits rigorous testing.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: NCBI Genbank accession numbers of D-loop sequences used for calculations of genetic distances. Asterisks indicate cases where no or insufficient sequences were available for the species used in the experiments.

| species	Genbank accession numbers
* *Pundamilia pundamilia/nyererei* * *Neochromis omnicaeruleus* * *Paralabidochromis rockkribensis/chilotes*	AF213528, AF213544, AF213545, AY930005, AY930006 *(Neochromis nigricans)* AF213540, AF213539, AF213525 *(Paralabidochromis chilotes)* AF213546, AF213548, AF213547, AF213529 *(Paralabidochromis plagiodon)*
* *Metriclima estherae*	AY930025 (*Metriaclima. zebra*), AF213620, AY911810, AY911811, AY911812 (*Metriaclima callanois*)
Astatotilapia calliptera	AF298938, AY911722, AY929977, AF298940, AF298939, AF298941, AY911723
Astatotilapia burtoni	AY929999, AF298905, AY929955, AF298906, AY930000, AY930001, AF298904
Protomelas taeniolatus	AF298963, AY913942, EF6475464

Supplementary Table 2: The amount of transgression and the variance explained by each PC axis for both F1 and F2 hybrids.

| cross type	Species crossed	TS on PC1 (%)	variance (%)	TS on PC2 (%)	variance (%)	TS on PC3 (%)	variance (%)	TS on PC4 (%)	variance (%)	TS on PC5 (%)	variance (%)	TS on PC6 (%)	variance (%)
	F1 hybrids												
1	*N. omni x P. pun*	20.62	31.95	24.20	24.65	0.00	15.71	0.00	11.22	0.00	9.12	0.00	7.35
2	*P. chil x P. ny*	0.00	30.55	134.46	22.78	0.00	17.84	0.00	12.01	0.00	9.35	1.80	7.47
3	*P. rock x P. pun*	55.79	36.40	24.49	19.51	18.36	12.95	43.89	11.44	0.00	10.31	0.00	9.38
4	*M. est x A. call*	0.00	45.32	0.00	31.31	0.98	13.86	0.00	9.51	-	-	-	-
5	*P. taen x A. call*	0.00	27.60	0.00	23.83	3.12	16.01	0.00	13.22	31.23	10.30	0.00	9.04
6	*A. burt x A. call*	0.00	42.19	0.00	25.85	38.60	12.89	6.03	10.03	0.00	9.04	-	-
7	*P. ny x A. call*	14.19	32.82	27.26	24.15	16.69	14.22	0.00	11.31	2.79	10.17	0.00	7.33
	F2 hybrids												
1	*N. omni x P. pun*	27.44	34.04	22.42	24.04	0.00	18.72	0.00	12.56	0.00	10.64		
2	*P. chil x P. ny*	8.46	38.03	0.00	20.65	13.85	13.85	8.01	11.63	4.33	8.17	0.00	7.67
3	*M. est x A. call*	28.36	53.37	0.00	20.72	22.66	10.50	0.55	8.78	9.56	6.64		
4	*P. taen x A. call*	26.28	50.95	0.00	18.08	8.05	12.59	0.00	10.29	0.00	8.10		
5	*A. burt x A. call*	54.37	54.73	14.91	19.80	37.97	15.88	3.50	9.59				
6	*P. ny x A. call*	60.93	0.61	4.43	0.04	7.56	0.08	5.20	0.05	1.85	0.02		

Supplementary Table 3: Test results from ANOVA with family as factor and PC scores as response variables. For n of families per cross type see Table 2.

cross type	Species crossed	PC1		PC2		PC3		PC4		PC5		PC6	
	F1 hybrids	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
1	*N. omni x P. pun*	20.30	< 0.001	9.15	< 0.001	6.91	< 0.001	7.15	< 0.001	2.52	0.061	0.75	0.525
2	*P. chil x P. ny*	0.02	0.892	6.07	0.018	2.43	0.127	0.14	0.713	1.53	0.223	3.91	0.055
3	*P. rock x P. pun*	46.50	< 0.001	98.32	< 0.001	0.68	0.507	4.68	0.011	0.55	0.578	0.63	0.532
4	*M. est x A. call*	7.35	< 0.001	9.70	< 0.001	1.91	0.080	1.26	0.280	-	-	-	-
5	*P. taen x A. call*	0.36	0.549	2.60	0.112	0.40	0.528	4.24	0.044	3.58	0.063	0.10	0.759
6	*A. burt x A. call*	10.09	< 0.001	1.13	0.348	0.30	0.828	23.24	<.0001	0.21	0.890	-	-
7	*P. ny x A. call*	3.30	0.003	7.09	< 0.001	8.48	< 0.001	4.07	< 0.001	2.24	0.034	2.44	0.022
	F2 hybrids	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
1	*N. omni x P. pun*	9.77	< 0.001	12.50	< 0.001	1.29	0.295	5.62	0.003	0.80	0.502	-	-
2	*P. chil x P. ny*	3.17	0.022	1.04	0.398	1.29	0.286	7.41	<.0001	11.24	<.0001	2.80	0.036
3	*M. est x A. call*	2.36	0.013	5.99	< 0.001	1.75	0.076	3.00	0.002	3.20	0.001	-	-
4	*P. taen x A. call*	1.76	0.122	1.21	0.315	4.10	0.002	1.21	0.314	2.23	0.052	-	-
5	*A. burt x A. call*	2.88	0.030	6.05	< 0.001	10.24	< 0.001	10.96	< 0.001	-	-	-	-
6	*P. ny x A. call*	2.87	0.013	4.50	0.001	0.21	0.981	4.05	0.001	2.78	0.015	-	-

a) *N. omni* x *P. pun*
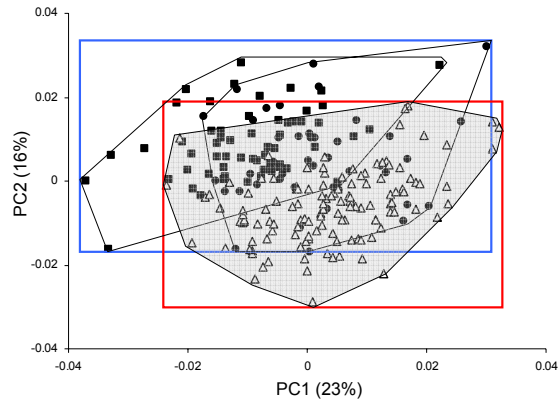
b) *P. chil* x *P. ny*
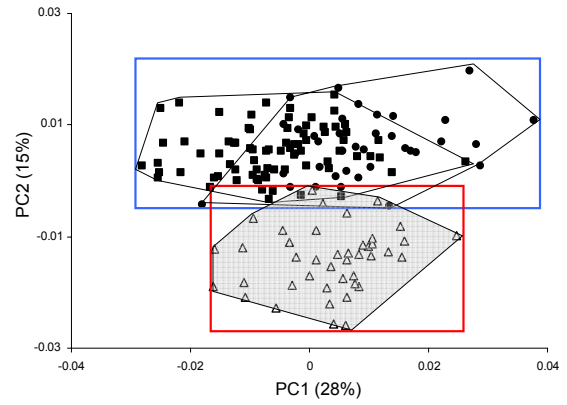
c) *P. rock* x *P. pun*
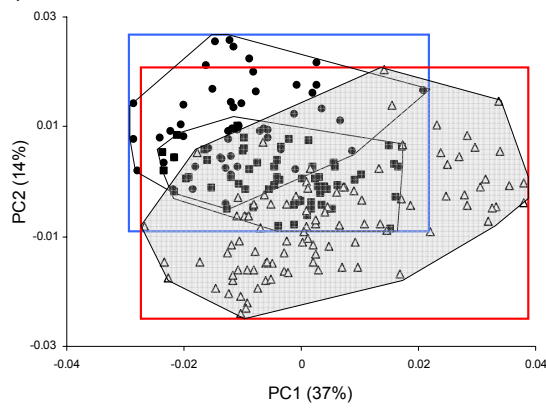
d) *M. est* x *A. call*
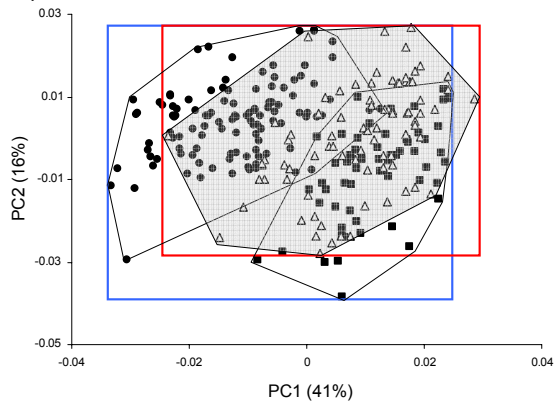
e) *P. taen* x *A. call*
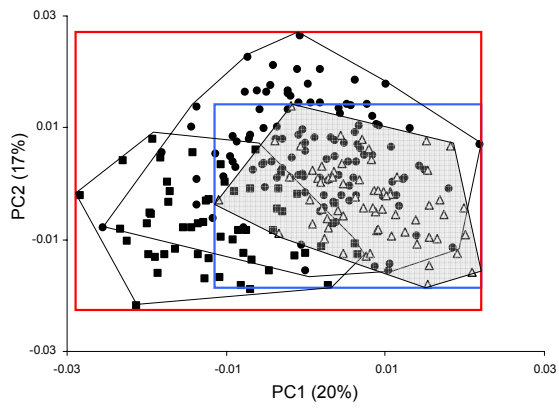
f) *A. burt* x *A. call*
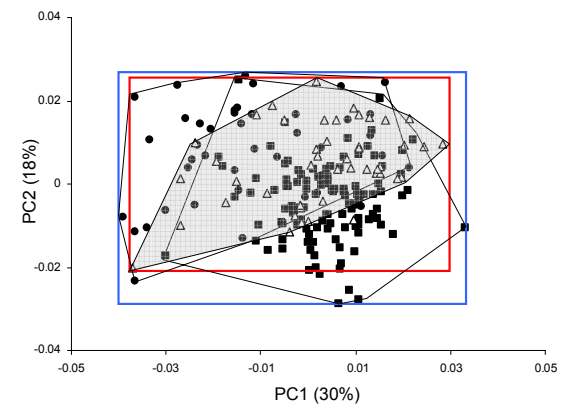
g) *P. ny* x *A. call*
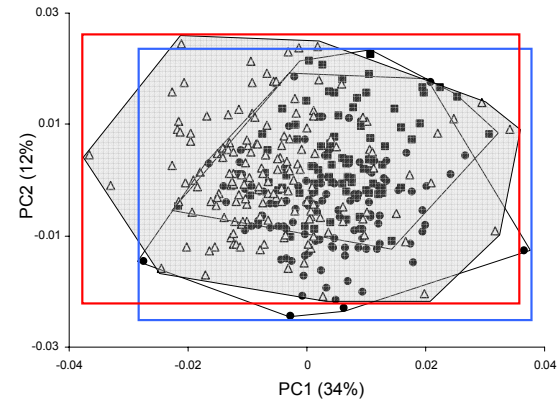
Supplementary Fig. 1: Results of principal component analyses using geometric morphometrics data to quantify the amount of transgression in shape of interspecific hybrids of haplochromine cichlids. Graphs show the distribution in morphospace of seven different F1 hybrid crosses and the corresponding homospecific crosses of species pairs with increasing genetic distance from smallest (a, b, c) to largest distance (g). Abbreviations of species names correspond to Table 2. Every data point represents one individual. Filled symbols indicate parental species, triangles indicate F1 hybrids. Blue squares encompass the phenotype range of the combined parental species; red squares represent the phenotype range of F1 hybrids. The percentage of variance explained by principal component 1 and 2 are shown in brackets. Note that the visualization of transgression is restricted to the first two axes of shape variation here, which is not (or not entirely) representative of the total amount of transgression found per cross type.
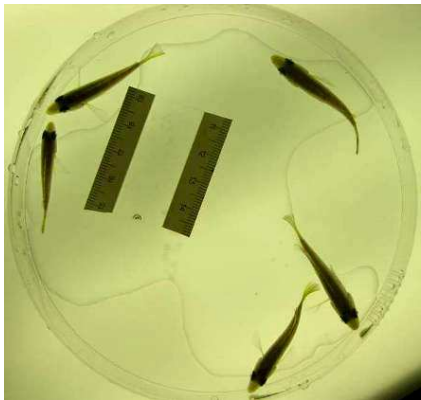
Chapter 4

Temporal patterns in the accumulation of reproductive incompatibilities between species of African cichlid fish

Rike B. Stelkens [1, 2, 3] *, Kyle A. Young [4], Ole Seehausen [1, 2]

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1. Division of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, Baltzerstr. 6, CH-3012 Bern, Switzerland
2. Department of Fish Ecology and Evolution, Centre of Ecology, Evolution and Biogeochemistry (CEEB), Eawag Swiss Federal Institute of Aquatic Science and Technology, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland
3. Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland
4. Universidad de Los Lagos, I-MAR, Camino a Chinquihue km. 6, Casilla 557, Puerto Montt, Chile

* Corresponding author: Rike.Stelkens@unil.ch; tel: +41 21 692 4249; fax: +41 21 692 4165

ABSTRACT

The rate at which reproductive isolation between species increases with divergence time remains poorly understood. Here, we measured the degree of premating isolation and hybrid inviability at four different ontogenetic stages spanning zygotes to adults in interspecific hybrids of 26 pairs of African cichlid species, representative of the entire haplochromine radiation. Our study is the first to use relaxed molecular clock calibrations to translate genetic distances into absolute age for comparing evolutionary rates of different components of reproductive isolation. We found that premating isolation accumulated faster initially but then changed little with increasing genetic distance between species. In contrast, postmating isolation between closely related species was almost completely absent but then accumulated rapidly causing complete hybrid inviability after 4.4/8.5/18.4 million years (depending on the molecular clock). Thus, the rate at which complete intrinsic incompatibilities arise in this system is orders of magnitude lower than rates of speciation within individual lake radiations. We conclude that extrinsic mechanisms (divergent ecological adaptations) prevent populations from interbreeding and are probably the most important stakeholders in maintaining cichlid species diversity, which is hence highly vulnerable to changes in the environments of the African Great Lakes. Dating the time window for successful hybridization in cichlid fish is interesting for yet another reason: quantifying the capacity to produce viable hybrids between allopatric, distantly related lineages is a key element when testing the feasibility of the 'hybrid swarm origin' model of adaptive radiation.

KEYWORDS: adaptive radiation, cichlid fish, reproductive isolation, speciation, divergence time, genetic distance, hybrid (in)viability, relaxed molecular clock.

AUTHORS' SUMMARY (*in layman's terms for submission to PLOS Biology*)

Both pre-and postmating isolation increase with the time of divergence between species. Premating isolation results from females and males of two different species being less likely to mate. Postmating isolation results mainly from genetic mismatches that reduce hybrid fitness. To estimate the rate at which these incompatibilities accumulate through time, biologists measure the isolation between species pairs that vary in genetic distance. We used African cichlid fish to address two shortcomings of this approach. First, we applied three different molecular clocks to translate genetic distances into absolute evolutionary time. Second, we estimated premating isolation and four life-stage specific components of postmating isolation covering the entire ontogeny of hybrid individuals. We found that premating isolation accumulated quickly but then changed little with time since divergence. Conversely, two components of postmating incompatibilities (fertilization rate and larval survival) decreased with time in a clock-like manner. Complete hybrid inviability was only reached after 4.4/8.5/18.4 million years, which is much longer than the speciation rates previously suggested for some cichlid radiations. We conclude that other factors, such as ecological adaptations, are more likely to maintain the large diversity of cichlid fish than the intrinsic mechanisms investigated here.

BLURB (*for submission to PLOS Biology*)

Evidence suggests hybridization may jump start adaptive radiation in African cichlid fish. We confirm the plausibility of this scenario by showing species separated by up to 18 my interbreed and produce viable hybrids.

INTRODUCTION

Speciation can be driven either by extrinsic mechanisms when ecological adaptations prevent populations from interbreeding, or by intrinsic mechanisms when genetic incompatibilities cause hybrid sterility or inviability. Because the former mechanism is thought to be more directly driven by selection than the latter, extrinsic mechanisms are thought to arise at greater pace and cause speciation more frequently. However, because extrinsic mechanisms may be much more easily reversible when the shape and magnitude of selection coefficients change, it is debatable which of the two mechanisms is more important in generating lasting patterns in species diversity. The large adaptive radiations of cichlid fish in East African lakes are a classical example where hundreds of species are thought to have arisen rapidly as a result of divergent selection. Much work has been directed at understanding the ecological causes of this rapid speciation. But to understand the resilience of the emergent species diversity to future environmental change, we also need to know the rate at which intrinsic incompatibilities arise in adaptive radiations.

Here, we address this question for the first time in the adaptive radiation of East African haplochromine cichlid fish. We present evolutionary rates of intrinsic incompatibilities measured across many species from several lakes, encompassing the phylogenetic depth and age of the entire haplochromine radiation. We show that intrinsic incompatibilities arise in a clock-like fashion. Juvenile (post hatching) mortality is the largest source of hybrid dysfunction, and it shows strongly clock-like behaviour. Incompatibilities at embryo (pre-hatching) stage on the other hand are weak and not clock-like. It is during post-hatching morphogenesis that many morphological species differences begin to manifest, indicating that intrinsic hybrid incompatibility may map predominantly to morphogenes, and may hence be a byproduct of natural selection. Nevertheless, the rate at which strong intrinsic incompatibilities arise in this adaptive radiation is many orders of magnitude lower than rates of speciation within individual lake radiations. We conclude that most of the staggering diversity of cichlid fish species must be maintained predominantly by extrinsic isolation, and hence be highly vulnerable to changes in the environments of the African Great Lakes.

Quantifying the rate at which different types of reproductive incompatibilities accumulate through time is a fundamental goal of speciation research (Otte and Endler 1989; Howard and Berlocher 1998; Turelli et al. 2001; Coyne and Orr 2004). Factors contributing to reproductive isolation can be classified into pre- and postmating incompatibilities (Coyne and Orr 2004). Premating reproductive isolation is the result of interactions between males and females that affect the likelihood of heterospecific matings (e.g. mating preferences, courtship location, timing and behaviour). Intrinsic postmating reproductive isolation results principally from the negative effects of Dobzhansky-Muller genetic incompatibilities on stage-variable survival rates to adulthood, e.g. fertilization success and hatching rates (Dobzhansky 1936; Muller 1942; Lynch 1991; Edmands 1999; Gharrett et al. 1999; Turelli and Orr 2000). Postmating isolation can also result from extrinsic ecological or sexual selection if hybrids are ecologically or reproductively less successful than individuals of the parent species (Hatfield and Schluter 1999; Vamosi and Schluter 1999; Naisbit et al. 2001; van der Sluijs et al. 2008b).

The geographical mode of speciation is predicted to affect the relative rate at which pre- and postmating incompatibilities accumulate. Because reinforcement can occur only in sympatry, premating incompatibilities are expected to accumulate faster in sympatric species than in allopatric species and faster than intrinsic postmating incompatibilities. In allopatry the two are hence expected to accumulate at similar rates because genetic drift should affect traits contributing to each form of incompatibility similarly (Dobzhansky 1951; Butlin 1989; Coyne and Orr 1989, 1997; Rundle and Schluter 1998; Jiggins et al. 2001; Nosil et al. 2003; Coyne and Orr 2004; Servedio 2004; Bridle et al. 2006). However, theory also suggests that premating incompatibility can accumulate rapidly in allopatry when species are characterized

by sexual dimorphism and strong sexual selection (Fisher 1930; Lande 1981; West-Eberhard 1983). For example, Mendelson (2003) found that premating incompatibility accumulated faster than hatching incompatibility among allopatric species pairs of sexually dimorphic darter fish (*Etheostoma*).

Coyne and Orr (1989) introduced the approach of comparing the degree of premating or postmating incompatibility to interspecific genetic distance, which when calibrated to absolute time using molecular clocks yields the well known 'speciation clock' (Edmands 2002; Bolnick and Near 2005; Bolnick et al. 2006). Despite important progress, the full potential of the approach has remained unrealized for two principal reasons. First, divergence times are typically estimated from molecular clocks that lack calibration to the fossil/geologic record and assume constant substitution rates through time and across lineages ((Coyne and Orr 1989; Knowlton et al. 1993; Coyne and Orr 1997; Foltz 1997; Sasa et al. 1998; Presgraves 2002; Tubaro and Lijtmaer 2002; Lijtmaer et al. 2003; Mendelson 2003; Moyle et al. 2004) ; for exceptions see Price and Bouvier 2002; Fitzpatrick 2004)). This uncertainty makes it difficult to interpret or compare the wide range (1.5 to 29 million years) of published estimates for the waiting time to complete incompatibility (Coyne and Orr 1989; Knowlton et al. 1993; Coyne and Orr 1997; Turelli and Begun 1997; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002; Lijtmaer et al. 2003; Bolnick and Near 2005)). Second, most studies have quantified the relationship between estimated divergence time and a measure of either premating isolation or postmating incompatibility. Most of the studies that have compared the two rates have done so for only a single measure of postmating incompatibility (Coyne and Orr 1989, 1997; Gleason and Ritchie 1998; Mendelson 2003; Moyle et al. 2004). We know of only two studies on plants that have compared a measure of premating isolation with measures of postmating incompatibility at multiple life stages (Scopece et al. 2007; Lowry et al. 2008). It may be inappropriate to draw conclusions about the rate at which postmating incompatibility accumulates based on fertilization or hatching success if genetic incompatibilities are expressed during later ontogenetic stages.

Here we present a study that addresses both these limitations. For 26 (mostly allopatric) species pairs of African haplochromine cichlid fishes we estimated the strength of premating isolation and quantified hybrid viability from fertilization through to adulthood for heterospecific and homospecific parental crosses. We quantify the relationship between five measures of incompatibility and time since isolation using three different molecular clocks: one linear clock calibrated to the biogeography of Lake Malawi (Sturmbauer et al. 2001), and two nonlinear clocks, one calibrated to the fossil record plus recent biogeographical events, the other to the break up of Gondwanaland plus recent biogeographical events (Genner et al. 2007).

Studying the rate at which reproductive compatibility declines in African cichlids is interesting for two reasons. First, African cichlids are typically sexually dimorphic with male breeding colour under sexual selection through female choice (Seehausen 1997; Knight and Turner 2004; Maan et al. 2004; Pauers et al. 2004; Stelkens et al. 2008; van der Sluijs et al. 2008a). For such systems theory predicts that premating compatability may be lost faster than intrinsic postmating compatability (Fisher 1930; Lande 1981; West-Eberhard 1983). Second, evidence suggests that some adaptive radiations of African cichlids may have been initiated through hybridization between distantly related lineages (Seehausen et al. 2003; Seehausen 2004). For example, the radiations of Lake paleo-Makgadikgadi (Joyce et al. 2005) and Lake Victoria (Seehausen et al. 2003) appear to be derived from multiple distantly related lineages (> 10 my for Lake Makgadikgadi, 8-15 my for Lake Victoria). Quantifying the rates at which different components of reproductive compatibility are lost between isolated cichlid lineages is important for understanding the role of hybridization in adaptive radiation.

METHODS
Genetic distance and divergence time estimates
Genetic distances between species pairs were calculated as uncorrected p-distances using all available D-loop sequences in NCBI GenBank (http://ncbi.nlm.nih.gov/Genbank/; Supplementary Table 1). All sequences were manually aligned following pairwise algorithm alignment in ClustalW (Thompson et al. 1997). Genetic distances were calculated in MEGA 4 (Kumar et al. 2004). For species without sequences, we used the sequences of closely related species (Supplementary Table 1). This is justified because all missing and replacement species were members of clades with incomplete mitochondrial DNA lineage sorting (the radiation of Lake Victoria and species within sub-clades of Lake Malawi Mbuna). Sequences were available for all species pairs with complete lineage sorting. When multiple sequences were available, we used the average p-distance (Mendelson 2003; Chapman and Burke 2007). All interspecific distances were corrected for intraspecific variation among haplotypes by subtracting average within-species distances (calculated as the mean of the intraspecific averaged distances of both species per cross) from mean between-species distances (Nei 1987; Mendelson 2003).

Divergence times were calculated using three molecular clocks. The first two were relaxed clocks calibrated using the cichlid fossil record and the fragmentation of Gondwanaland (Genner et al. 2007). We used the power functions of Genner et al. (Fig. 5d in their paper) which show divergence in the D-loop region of mitochondria is rapid for first one million years then declines until reaching a stable baseline substitution rate at two million years. Similar nonlinear patterns have been found in birds and primates (Ho and Larson 2006; Ho 2007). The third clock was internally calibrated using the age of Lake Malawi (Sturmbauer et al. 2001).

Breeding protocol
All fish used in the experiment were derived from laboratory populations maintained in our breeding facility at Eawag, Kastanienbaum, Switzerland. Hybrid families were created between October 2005 and August 2007 in aquaria (100 x 40 x 40 cm) stocked with five to twenty females and a single male. A shelter of three stones served as the male territory. Fish were fed daily with dry food and twice a week with a blend of shrimp, peas and *Spirulina* powder. Light regime was 12L:12D and water temperature was 24 - 26 °C. All the study species are maternal mouth-brooders. Tanks were checked daily for brooding females which were left in the aquarium for four days to avoid premature release of the eggs. Eggs were then collected by holding the female vertically in the water and gently opening her mouth. The males and females that spawned were replaced with new individuals so only one family was bred from any one female and any one male.

Measuring premating isolation
The experimental unit of observation for measuring premating isolation was the aquarium. We used the proportion of females that did not spawn (1- number of spawned females/total number of females) to estimate the strength of premating reproductive isolation, with values near '1' indicating nearly complete premating isolation. If crosses were made in more than one tank, we used the average across tanks. Breeding experiments were terminated once enough hybrid families were obtained or when it became clear that no more spawnings would occur. Hence, trials lasted for different lengths of time (from 24 to 593 days). To test if the duration of trials affected our estimate of premating isolation, we regressed the isolation index on trial duration. We also regressed trial duration on genetic distance.

We know from experience that even in homospecific crosses, rarely do all females in a tank breed regardless how much time they are given. To control for 'homospecific premating isolation', we estimated the proportion of females that did not spawn with conspecific males

in three different species of *Pseudocrenilabrus* from Lake Mweru, Zambia/DCR (Stelkens & Seehausen, submitted), and two species of *Pundamilia* from Lake Victoria (Stelkens et al. 2008; C.J. Allender & O. Seehausen, unpublished data). In these experiments, females were tested for assortative mating between conspecific males and males of closely related species. We used the same calculation as above, but subtracted the number of females that spawned with the heterospecific male (1 – number of spawned females /(total number of females - females spawning with a heterospecific male)). We use the mean isolation index from these five crosses as a homospecific comparison (i.e. premating isolation at ~zero genetic distance) to the levels of isolation observed in our interspecific mating trials.

The proportion of non-breeding females was regressed on each of the three estimates of divergence time. The homospecific control data point was not included in the regression analyses but is shown in the figures for comparison and is used to facilitate comparisons with indices of hybrid inviability. We used stepwise general linear models in S-PLUS 7.0 (year and company?) with linear and quadratic terms, using AIC to select the best model.

Measuring hybrid inviability
We measured hybrid inviability for four life history stages: 1) fertilization, 2) hatching, 3) survival rate at 14 days, and 4) survival at day 180 which in cichlids corresponds to early adulthood and sexual maturity.

Fertilization failure per clutch was calculated as 1- (number of fertilized eggs / total number of eggs) five days after mating had occured (fertilized eggs are easily distinguished by colour and texture). Fertilized eggs were transferred to identical egg tumblers (description available from the corresponding author). Tumblers were checked daily and hatching mortality per clutch was calculated as 1- (number of hatched / number of fertilized eggs) during the first 14 days. Dead fry were removed from the tumblers daily and we calculated 14-day mortality as 1- (number of survivors on day 14/ number of hatchlings). On day 15 all fry were moved to aquaria (20 x 40 x 20 cm), maintained at the same light and temperature conditions as above. On day 30 the fish were transferred into larger aquaria (50 x 40 x 30 cm) at a maximum density of 20 individuals per aquarium. Fish were fed daily at a constant per fish rate. For each clutch we calculated 180-day mortality as 1- (number of survivors on day 180 / number of individuals on day 30). Finally, we calculated the total (cumulative) hybrid inviability as 1- (number of survivors on day 180 / total number of eggs laid). All measures of hybrid inviability were regressed against divergence time using the same stepwise procedure as above (except for cumulative inviability where a logarithmic model provided the best fit).

Calculating incompatibility
To control for homospecific levels of fertilization failure and mortality, we raised three clutches of each parental species using the identical procedure and measured the same four components of postmating isolation. We calculated incompatibility for each of the four measures of hybrid inviability and cumulative inviability using equation (1) (Bolnick and Near 2005). Values of the two homospecific cross types were averaged. Since most cross types were replicated (Supplementary Table 2), we used the average across clutches for each cross type (reciprocal crosses pooled).

equation (1) $incompatibility = 1 - \dfrac{\%\,success\ in\ heterospecific\ crosses}{\%\,success\ in\ homospecific\ crosses}$

Incompatibilities approaching 1 indicate nearly complete failure at a life history stage, whereas those close to zero indicate hybrids have similar fertilization/hatching/survival rates as homospecifics. The incompatibility data were analyzed using the same procedure as above.
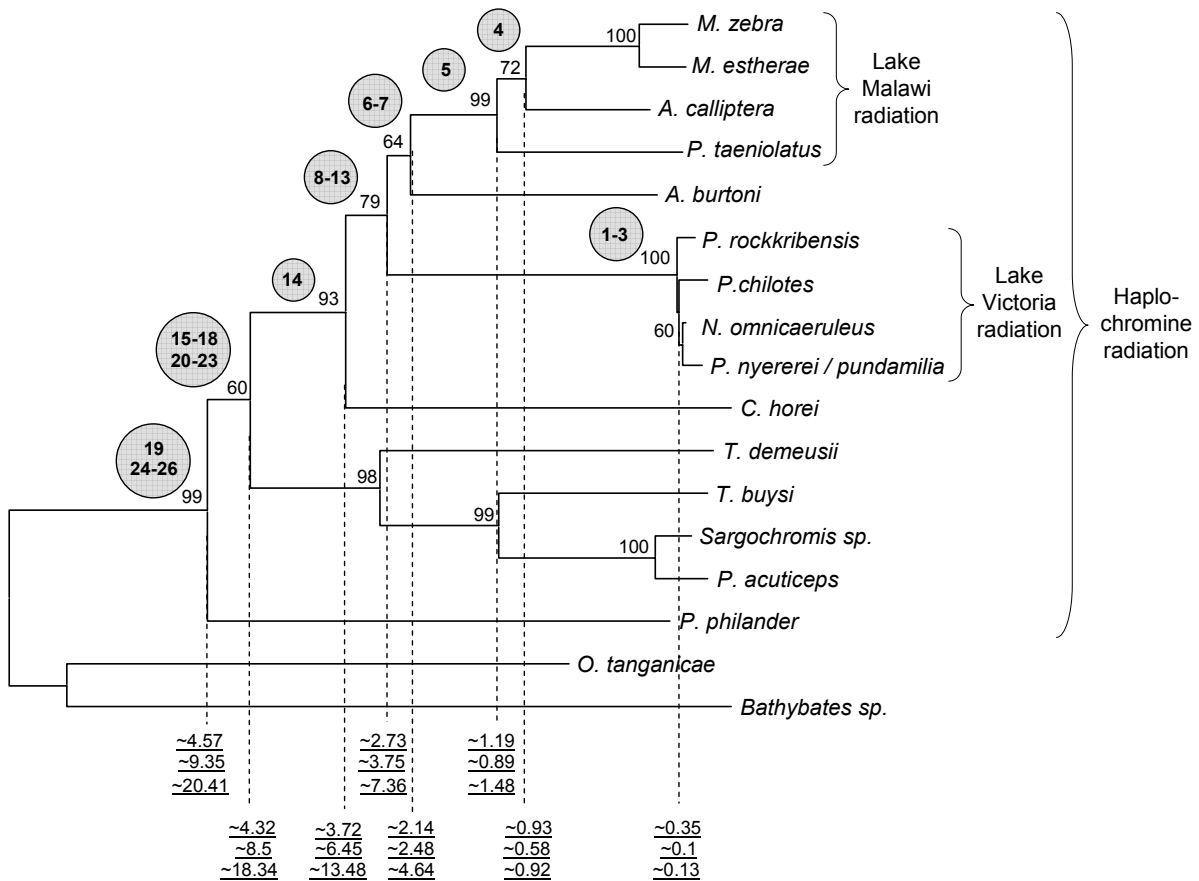
Fig. 1: Neighbour joining estimate of the phylogeny of the entire East African radiation of haplochromine cichlid fish, featuring the 16 species used in this study to make interspecific crosses. Only values > 50% are shown for 1000 bootstrap pseudoreplicates. Numbers in circles correspond to the hybrid crosses described in Supplementary Table 2 and indicate the eight nodes in the phylogeny for which independent contrasts in reproductive isolation and divergence time were calculated. Cross 19 is not in consecutive order because in Supplementary Table 2 it was assigned a slightly smaller p-distance than the other crosses spanning that node. This was due to the high intraspecific genetic distances found in both its parental species, which was subtracted from the interspecific distance. The clades representing the endemic radiations of Lake Malawi (~500 spp), Lake Victoria (~500 spp) and the entire East African radiation are indicated. Divergence times in millions of years are shown underneath the tree. Upper values indicate node ages calculated from a linear, internally calibrated clock using only recent biogeographical events, middle values show node age estimates from a non-linear clock based on the fossil record plus recent biogeographical events, lower values show node ages from a non-linear clock based on the break up of Gondwanaland plus recent biogeographical events. Values represent average divergence time estimates of all crosses spanning that node. The closely related sister species *P. nyererei* and *P. pundamilia* were treated as one taxon in this phylogeny.

Controlling for the non-independence of pairwise relationships

To estimate the phylogenetic relationship of the 16 species we calculated p-distances using mitochondrial D-loop sequences (903bp) and performed neighbour joining analysis with 1000 bootstrap pseudoreplicates in MEGA (Kumar et al. 2004) (Fig. 1). We then calculated independent contrasts for all test variables across each node in the phylogeny (Felsenstein 1985). Following Coyne and Orr (1989, 1997) we calculated means for each node by averaging the values for all species pairs that span the node.

Contrasts were calculated for 1) the strength of premating isolation and hybrid inviability (without controlling for inviability of homospecific crosses); 2) the incompatibility of hybrid crosses controlling for the inviability observed in both corresponding homospecific crosses. The first analysis was used to compare the rates at which pre- versus postmating

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isolation accumulate. The independent contrast data were analyzed following the same stepwise procedure as applied to the raw data.

RESULTS

We obtained 26 cross types from parents of 16 haplochromine cichlid species with divergence times ranging from several thousand years to 6.6/10/22 million years (from here forward divergence time estimates are given in the following order: the internal calibration/fossil calibration/Gondwana calibration). Supplementary Table 2 reports crosses with genetic distances, divergence times and sample sizes for the different pre- and postmating measures.

Premating isolation

The proportion of females that spawned did not depend on trial duration ($R^2 = 0.02$; $F_{1,57} = 1.11$, $p = 0.3$), and trial duration was not related to the genetic distance between the parents ($R^2 = 0.02$; $F_{1,57} = 0.86$, $p = 0.37$, Supplementary Table 3).

The proportion of non-breeding females increased significantly with divergence time and was best explained by a linear model for all three clock estimates (Fig. 2a). The average proportion of non-breeding females in homospecific crosses of three *Pseudocrenilabrus* and two *Pundamilia* species was 0.44 ± 0.15. To obtain an intercept for the relationship between premating isolation and divergence time, we subtracted the homospecific value (0.44) from all heterospecific values and re-ran the analysis (all results in Table 1a). The phylogenetically independent contrasts yielded nearly identical results (Fig. 2b). The proportion of non-breeding females increased significantly with node age based an all three molecular clocks. Again, the homospecific value was subtracted from all data points to obtain an estimate of the intercept (Table 1b). Based on the regression model (using contrast data) premating isolation reached 50% after ca. 1.4/1.0/1.7 my and complete isolation well after > 4.8/10/22 my (Fig. 2a, b).

The observed heterospecific intercept of the relationship between premating isolation and genetic distance was not significantly different from the homospecific values regardless of the molecular clock used (using raw data: $t = 0.54/0.45/0.43$, $p = 0.59/0.66/0.67$; using contrast data: $t = 0.46/0.36/0.35$, $p = 0.64/0.72/0.73$). This indicates that the estimate we obtained for homospecific crosses (basically representing a data point at zero genetic distance) was in agreement with the intercept we obtained from the model.
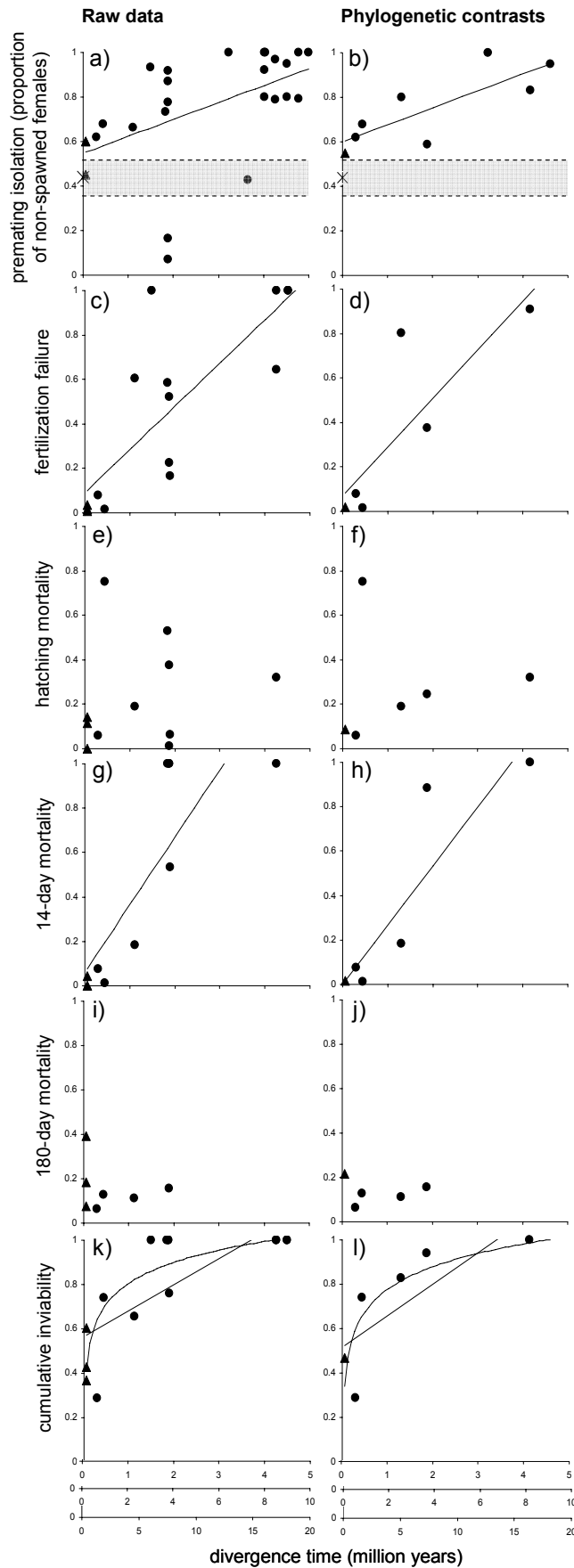
Fig. 2: Accumulation of premating isolation and hybrid inviabilities as a function of divergence time based on three different molecular clocks. Upper X-axis shows divergence times calibrated to the biogeography of Lake Malawi; middle x-axis shows divergence times calibrated to the fossil record plus recent biogeographical events; lower x-axis shows divergence times calibrated to the break up of Gondwanaland plus recent biogeographical events. Premating isolation was calculated from data on the proportion of non-breeding females. Hybrid inviability was calculated from data on fertilization, hatching, survival after 14 days, and survival after 180 days. Cumulative inviability (k, l) contains all four life history stages. Left column: Each data point represents a different hybrid cross. Right column: Each data point represents one phylogenetically independent contrast in reproductive isolation and in divergence time. Triangles indicate sympatric species crosses. Asterisks indicate the mean strength of premating isolation in homospecific crosses. The grey zones in a) and b) indicate the 95% confidence interval around the homospecific isolation mean. Regression lines are only shown where significant. In k) and l) both logarithmic and linear regression lines are shown for better comparison with the premating data. Results of all regression analyses can be found in Table 1.

Raw data   **Phylogenetic contrasts**
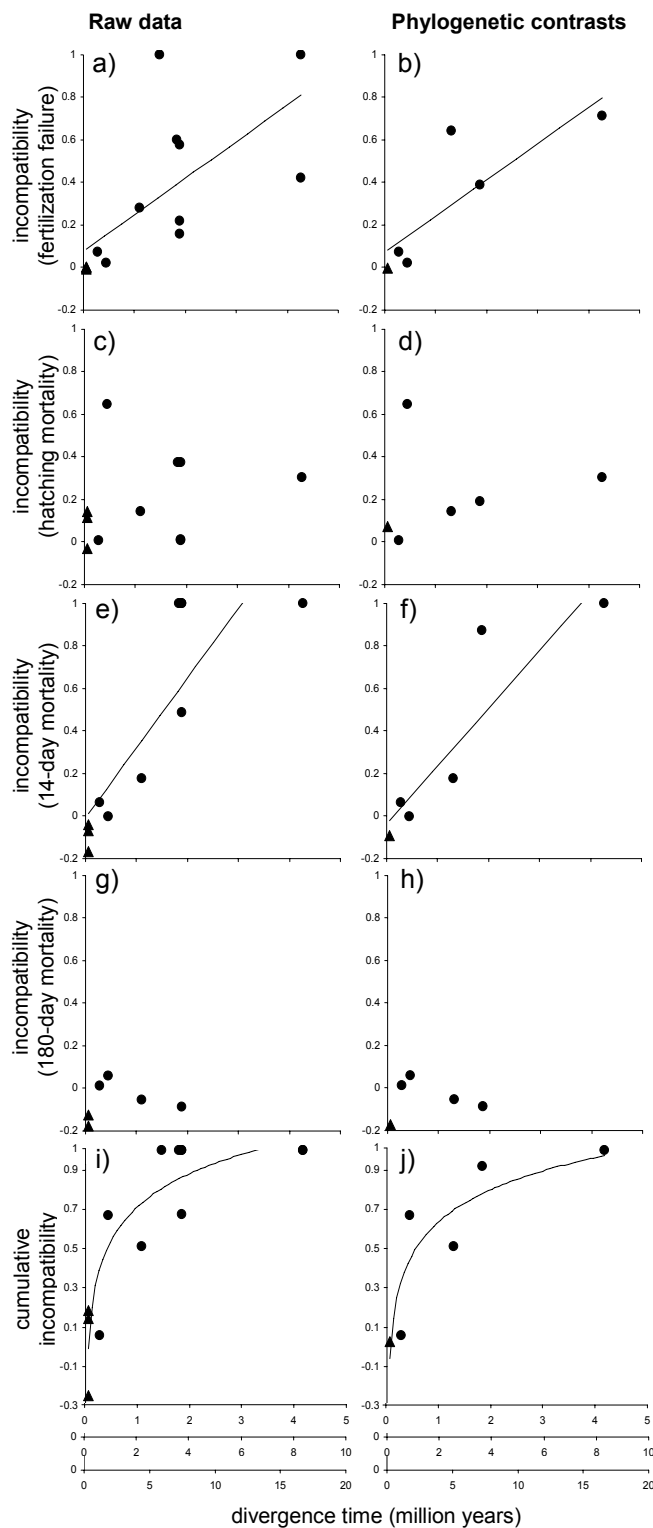
Fig. 3: Accumulation of incompatibility of hetero- and homospecific crosses as a function of divergence time based on three different molecular clocks. Upper X-axis shows divergence times calibrated to the biogeography of Lake Malawi; middle x-axis shows divergence times calibrated to the fossil record plus recent biogeographical events; lower x-axis shows divergence times calibrated to the break up of Gondwanaland plus recent biogeographical events. Incompatibilities were calculated from hybrid inviability data (Fig. 2), controlled for the inviability found in homospecific crosses. Left column: Each data point represents a different hybrid cross. Right column: Each data point represents one phylogenetically independent contrast in incompatibility and in divergence time. Incompatibilities below 0% indicate that hybrids have higher viability than the two corresponding homospecific crosses. Open circles indicate sympatric species crosses. Regression lines are only shown where significant. Results of all regression analyses can be found in Table 2.

divergence time (million years)

Hybrid inviability

We obtained 105 clutches from 15 crosses with parental species divergence times ranging from several thousand to 6.2/9/19.6 my.

Hybrid inviability significantly increased with divergence time for two of the four life history stages (Table 1a, Fig. 2 c, e, g, i, k). Fertilization failure and 14-day mortality increased linearly with divergence time, whereas hatching mortality and 180-day mortality

were unrelated to divergence time. Cumulative inviability accumulated at a decelerating rate and was best explained by a logarithmic function (Table 1a, Fig. 2 k).

Again, the raw data and phylogenetic contrasts yielded nearly identical results (Table 1b, Fig. 2d, f, h, j, l). Inviability resulting from fertilization failure and 14-day mortality was negligible for species pairs separated for up to 1.2/0.9/1.5 my, reached 50% at ca. 2.8/4.0/7.9 my, and resulted in nearly complete isolation by 4.3/8.3/17.9 my.

Juvenile survival (14-day mortality) determined hybrid inviability. All fish that successfully hatched from parents with $\geq$ 4.4/8.5/18.4 my divergence time died within 14 days (crosses 22 and 21, Supplementary Table 2). In clutches from parents with $\geq$ 2.7/3.8/7.4 my divergence time, 49% of the hatchlings survived beyond 14 days and were successfully raised to adulthood (i.e. until 180 days, cross 12). Complete fertilization failure was observed after 4.5/9.0/19.6 my divergence (crosses 22 and 23). After 4.4/8.5/18.4 my, on average 48% of eggs per clutch were successfully fertilized, of which 68% hatched (but then died within 14 days, crosses 22 and 21).

Comparing the accumulation rates of premating isolation and hybrid inviability

After subtracting the homospecific value from all interspecific data points of premating isolation (using contrast data), we used *t*-tests to compare the slopes and intercepts of the relationships between premating isolation and hybrid inviability on divergence time (computed as the difference between the slopes/intercepts, divided by the standard error of the difference between the slopes/intercepts). The intercept for premating isolation was higher than that for fertilization failure, but the difference was significant only for the internally calibrated clock regressions ($t = 3.17/1.67/1.37$, $p = 0.007/0.12/0.19$, using data on phylogenetic contrasts). The intercept was significantly higher than that for 14-day mortality with all three molecular clocks ($t = 4.84/3.27/2.86$, all $p < 0.05$). Hybrid inviability increased at a significantly faster rate than premating isolation for both fertilization failure ($t = 5.93/5.35/5.14$, all $p < 0.001$) and 14-day mortality ($t = 8.0/7.9/7.6$, all $p < 0.001$). Fertilization failure increased by 24/11/5 % per million years (my), 14-day mortality by 29/13/6 % per my, and premating isolation by 9/4/2 % per my.

A non-linear, logarithmic model provided the best fit for total inviability but prevented us from comparing the accumulation rates and intercepts of premating isolation and cumulative inviability. Hence, in addition to the logarithmic regression we also fit a linear model here to provide a meaningful comparison with the premating data (Table 1, Fig. 2 k, l). The intercepts of premating isolation and cumulative inviability did not significantly differ ($t = 0.06/-0.08/-0.10$, $p = 0.96/0.93/0.92$), but total hybrid inviability increased at a significantly faster rate than premating isolation (*t*-tests on slopes: $t = 3.74/3.03/2.84$, all $p = 0.002/0.01/0.013$) with 16/7/3 % per my.

Together, these results suggest that premating isolation accumulates faster initially but then changes slowly with increasing genetic distance between species only reaching completion after >4.8/10/22 my. In contrast, postmating isolation is weak between closely related species, but then increases relatively rapidly with complete hybrid inviability occurring at 4.4/8.5/18.4 million years. This suggests that successful hybridization is ultimately limited by hybrid inviability rather than by premating isolation.

Postmating incompatibility

Consistent with the uncorrected inviability data, only fertilization failure and 14-day mortality increased significantly with node age with a linear model providing the best fit while hatching and 180-day mortality were not affected (Table 2a, Fig. 3, left column). Cumulative incompatibility increased rapidly first but then at decelerating rate (Table 2a, Fig. 3i). Analysis of the phylogenetic contrasts yielded similar results (Table 2b, Fig. 3, right column).

Incompatibility generally started off very low, in the case of 14-day and 180-day

mortality even below 0%, suggesting that hybrids between the most closely related species (crosses within Lake Victoria) had higher survival rates than the corresponding homospecific crosses. Incompatibilities reached 50% after ca 2.8/4.0/7.9 my (14-day survival), also confirming the above findings. As with the inviability data, 14-day survival reached complete incompatibility first after a node age of 4.4/8.5/18.4 my.

DISCUSSION

Reproductive compatibility between species typically decreases with time since geographic isolation, leading to the controversial assumption (Edmands 2002; Bolnick and Near 2005; Bolnick et al. 2006) that the progress toward speciation is predictable from the length of time spent in allopatry (Coyne and Orr 1998; Orr and Turelli 2001). There are however many problems with this assumption. Some are methodological, perhaps the most crucial of them being that genetic distance is often used as a surrogate for divergence time predicting that mutations accumulate constantly through time and across lineages, a difficulty that may be overcome by age calibrations with relaxed molecular clocks. Another problem is the conceptual misunderstanding that speciation is only complete when hybrids are inviable or infertile whereas other forms of isolation could already have impeded gene flow at an earlier stage of divergence.

The difficulty of comparing the rate at which incompatibilities accumulate is highlighted by the wide range of reported estimated of the time to hybrid inviability. Divergence times of 1.5-3.5 my seem sufficient to cause strong hybrid inviability in *Drosophila* (Coyne and Orr 1989), anurans (Sasa et al. 1998), sea stars (Foltz 1997), sea urchins (Lessios and Cunningham 1990) and shrimps (Knowlton et al. 1993). Viable hybrids have been observed between mammalian taxa separated by 8 my (Fitzpatrick 2004). In centrarchid fish the successful production of hybrids does not seem to cease until 29 my (Bolnick and Near 2005), and birds seem able to successfully hybridize up to 55 my, although fertility of these crosses was not confirmed (Prager and Wilson 1975; Cooper and Penny 1997; Price and Bouvier 2002). Clearly, we need studies from many more taxa in order to establish more general rules for how hybrid incompatibility changes through time.

Here, we tested at which rate the 'incompatibility clock' ticks in African cichlid fish, using hybrid crosses from 26 mostly allopatric species pairs covering absolute divergence times from several thousand years to 6.6/10/22 million years (using three different molecular clocks: a linear clock, only calibrated to recent biogeographical events (Sturmbauer et al. 2001), and two non-linear clocks, one calibrated to the fossil record plus recent biogeographical events, the other to the break up of Gondwanaland plus recent biogeographical events (Genner et al. 2007)). Premating incompatibility was estimated from the proportion of females that spawned with heterospecific males. Postmating incompatibility was measured from hybrid inviability rates in the lab. Because the genetic basis of (in)viability is usually multifarious we measured several different elements of intrinsic postmating compatibility at various developmental stages. Although taking several measurements from the embryonic stage to adulthood reduces the risk of underestimating overall incompatibility, only very few studies have tracked hybrid viability after the embryonic or larval stage.

Table 1 a) Results of regression analyses testing the strength of premating isolation, four different measures of hybrid inviability, and cumulative inviability (containing all four inviability components) against divergence time (without controlling for isolation in homospecific crosses). Only results of the best fitting models are presented. Although the logarithmic model fitted the cumulative measure best, linear models are also shown for better comparison with the premating data. For each regression we present the estimated intercept with standard error (SD) after subtraction of the homospecific control value (0.44), the estimated slope with SD, R^2-values, F-values and significance of whole model effects. Non-significant models are in italics. The first value per cell is calculated from a linear, internally calibrated clock using only recent biogeographical events; middle values are calculated from a non-linear clock based on the fossil record plus recent biogeographical events, and the third value is calculated from a non-linear clock based on the break up of Gondwanaland plus recent biogeographical events. Sample sizes are n = 26 cross types for premating isolation, n = 15 for fertilization, n = 11 for hatching and 14-day survival, n = 7 for 180-day survival, and n = 15 for the cumulative measure; b) same as a) but regressing phylogenetically independent contrasts in premating isolation and hybrid inviability against contrasts in node age. Number of contrasts were n = 8 for premating isolation, n = 6 for fertilization, hatching and 14-day survival, n = 5 for 180-day survival, and n = 6 for the cumulative measure.

a) Raw data	Best model	Intercept (SD)	Slope (SD)	R^2	F	p
premating isolation	linear	0.06 (0.11)/ 0.11 (0.08)/ 0.12 (0.08)	0.08 (0.03)/ 0.04 (0.01)/ 0.02 (0.0)	0.22/ 0.24/ 0.24	6.61/ 7.34/ 7.44	0.017/ 0.013/ 0.012
inviability (fertilization failure)	linear	-0.07 (0.11)/ 0.09 (0.1)/ 0.12 (0.09)	0.22 (0.04)/ 0.1 (0.02)/ 0.04 (0.0)	0.7/ 0.67/ 0.65	29.66/ 25.84/ 24.4	0.000/ 0.000/ 0.000
inviability (hatching mortality)	linear	*0.16 (0.13)/ 0.19 (0.11)/ 0.2 (0.1)*	*0.04 (0.06)/ 0.02 (0.03)/ 0.01 (0.01)*	*0.05/ 0.03/ 0.03*	*0.49/ 0.31/ 0.27*	*0.50/ 0.59/ 0.61*
inviability (14-day mortality)	linear	-0.14 (0.13)/ 0.06 (0.1)/ 0.09 (0.12)	0.31 (0.06)/ 0.15 (0.03)/ 0.07 (0.02)	0.77/ 0.7/ 0.67	30.81/ 21.21/ 18.25	0.000/ 0.001/ 0.002
inviability (180-day mortality)	linear + quadratic	*0.29 (0.12)/ 0.21 (0.08)/ 0.2 (0.07)*	*linear: -0.24 (0.24)/ -0.13 (0.14)/ -0.06 (0.07)* *quadratic: 0.07 (0.08)/ 0.03 (0.04)/ 0.01 (0.01)*	*0.24/ 0.18/ 0.16*	*0.62/ 0.43/ 0.38*	*0.58/ 0.68/ 0.71*
cumulative inviability	logarithmic	0.66 (0.04)/ 0.71 (0.04)/ 0.65 (0.04)	0.24 (0.04)/ 0.13 (0.02)/ 0.12 (0.02)	0.76/ 0.72/ 0.72	34.32/34.07/ 34.13	0.000/ 0.000/ 0.000
	linear	0.45 (0.07)/ 0.57 (0.07)/ 0.59 (0.07)	0.14 (0.03)/ 0.06 (0.01)/ 0.03 (0.01)	0.69/ 0.58/ 0.54	29.14/17.67/ 15.57	0.000/ 0.001/ 0.002
b) Phylogenetic contrasts						
premating isolation	linear	0.1 (0.07)/ 0.16 (0.06)/ 0.17 (0.06)	0.09 (0.02)/ 0.04 (0.01)/ 0.02 (0.01)	0.66/ 0.65/ 0.64	11.89/ 11.21/ 10.87	0.014/ 0.016/ 0.017
inviability (fertilization failure)	linear	-0.1(0.17)/ 0.07 (0.14)/ 0.1 (0.14)	0.24 (0.07)/ 0.11 (0.04)/ 0.05 (0.02)	0.75/ 0.7/ 0.68	11.88/ 9.17/ 8.38	0.026/ 0.039/ 0.044
inviability (hatching mortality)	linear	*0.23 (0.2)/ 0.26 (0.16)/ 0.26 (0.15)*	*0.02 (0.09)/ 0.01 (0.04)/ 0.0 (0.02)*	*0.01/ 0.01/ 0.01*	*0.06/ 0.03/ 0.02*	*0.82/ 0.88/ 0.89*
inviability (14-day mortality)	linear	-0.2 (0.16)/ 0.0 (0.13)/ 0.03 (0.13)	0.29 (0.07)/ 0.13 (0.03)/ 0.06 (0.02)	0.82/ 0.81/ 0.8	18.53/ 17.05/ 15.56	0.013/ 0.015/ 0.017
inviability (180-day mortality)	linear + quadratic	*0.28 (0.08)/ 0.19 (0.06)/ 0.18 (0.06)*	*linear: -0.24 (0.12)/ -0.11 (0.09)/ -0.05 (0.05)* *quadratic: 0.07 (0.04)/ 0.03 (0.02)/ 0.01 (0.01)*	*0.65/ 0.43/ 0.36*	*1.87/ 0.74/ 0.57*	*0.35/ 0.57/ 0.64*
cumulative inviability	logarithmic	0.61 (0.08)/ 0.67 (0.07)/ 0.6 (0.08)	0.26 (0.09)/ 0.15 (0.05)/ 0.13 (0.04)	0.69/ 0.69/ 0.69	8.82/ 8.7/ 8.73	0.041/ 0.042/ 0.042
	linear	0.39 (0.12)/ *0.52 (0.11)/ 0.54 (0.11)*	0.16 (0.05)/ *0.07 (0.03)/ 0.03 (0.01)*	0.71/ *0.6/ 0.58*	9.56/ *6.11/ 5.42*	0.04/ *0.07/ 0.08*

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Table 2 a) Results of regression analyses testing the accumulation of incompatibility against divergence time (controlling for incompatibility in homospecific crosses). Only results of the best fitting models are presented. Although the logarithmic model fitted the cumulative measure best, linear models are also shown for better comparison with the other results. For each regression we present the estimated intercept with standard error (SD), the estimated slope with SD, R^2-values, F-values and significance of whole model effects. Non-significant models are in italics. The first value per cell is calculated from a linear, internally calibrated clock using only recent biogeographical events; middle values are calculated from a non-linear clock based on the fossil record plus recent biogeographical events, and the third value is calculated from a non-linear clock based on the break up of Gondwanaland plus recent biogeographical events. Sample sizes are n = 13 cross types for fertilization, n = 11 for hatching and 14-day survival, n = 7 for 180-day survival; and n = 13 for the cumulative measure. b) same as a) but testing phylogenetically independent contrasts in the accumulation of incompatibility against contrasts in node age. Number of contrasts were n = 6 for fertilization, hatching and 14-day survival, n = 5 for 180-day survival, and n = 6 for the cumulative measure.

a) Raw data	Best model	Intercept (SD)	Slope (SD)	R^2	F	p
incompatibility (fertilization failure)	linear	-0.06 (0.13)/ 0.08 (0.11)/ 0.1 (0.1)	0.19 (0.05)/ 0.09 (0.03)/ 0.04 (0.01)	0.52/ 0.47/ 0.45	12.08/ 9.6/ 8.87	0.005/ 0.01/ 0.013
incompatibility (hatching mortality)	linear	*0.12 (0.12)/ 0.15 (0.09)/ 0.15 (0.09)*	*0.04 (0.05)/ 0.02 (0.03)/ 0.0 (0.0)*	*0.06/ 0.04/ 0.04*	*0.54/ 0.41/ 0.39*	*0.48/ 0.54/ 0.55*
incompatibility (14-day mortality)	linear	-0.22 (0.13)/ -0.0 (0.12) / 0.03 (0.13)	0.34 (0.06)/ 0.17 (0.04)/ 0.07 (0.02)	0.8/ 0.71/ 0.67	35.08/ 21.88/ 18.56	0.000/ 0.001/ 0.002
incompatibility (180-day mortality)	linear + quadratic	*-0.29 (0.06)/ -0.16 (0.05)/ -0.15 (0.05)*	*linear: 0.4 (0.1)/ 0.2 (0.1)/ 0.09 (0.05)* *quadratic: -0.12 (0.04)/ -0.05 (0.03)/-0.01 (0.0)*	*0.76/ 0.51/ 0.43*	*6.46/ 2.06/ 1.53*	*0.056/ 0.24/0.32*
cumulative incompatibility	logarithmic	0.44 (0.06)/ 0.54 (0.06)/ 0.43 (0.06)	0.43 (0.06)/ 0.24 (0.04)/0.22 (0.03)	0.81/ 0.81/ 0.81	46.5/ 46.39/ 46.38	0.000/ 0.000/0.000
	linear	0.05 (0.12)/ 0.26 (0.12)/ 0.3 (0.05)	0.27 (0.05)/ 0.12 (0.03)/ 0.05 (0.01)	0.72/ 0.57/ 0.53	28.35/ 14.48/ 12.28	0.000/ 0.003/0.005
b) Phylogenetic contrasts						
incompatibility (fertilization failure)	linear	-0.08 (0.12)/ 0.07 (0.11)/ 0.09 (0.11)	0.19 (0.05)/ 0.09 (0.03)/ 0.04 (0.01)	0.79/ 0.7/ 8.68	14.62/ 9.4/ 8.32	0.019/ 0.037/ 0.044
incompatibility (hatching mortality)	linear	*0.18 (0.18)/ 0.2 (0.14)/ 0.2 (0.14)*	*0.03 (0.08)/ 0.01 (0.04)/ 0.0 (0.02)*	*0.03/ 0.02/ 0.02*	*0.12/ 0.09/ 0.08*	*0.74/ 0.78/ 0.79*
incompatibility (14-day mortality)	linear	-0.25 (0.15)/ -0.04 (0.13)/ -0.0 (0.13)	0.3 (0.06)/ 0.14 (0.03)/ 0.06 (0.02)	0.85/ 0.82/ 0.8	22.83/ 17.68/ 15.7	0.009/ 0.014/ 0.017
incompatibility (180-day mortality)	linear + quadratic	*-0.28 (0.09)/ -0.12 (0.09)/ -0.1 (0.09)*	*linear: 0.42 (0.14)/ 0.18 (0.14)/ 0.08 (0.08)* *quadratic: -0.13 (0.04)/ -0.05 (0.04)/-0.01 (0.0)*	*0.822/ 0.47/ 0.37*	*4.62/ 0.88/ 0.59*	*0.18/ 0.53/0.63*
cumulative incompatibility	logarithmic	0.37 (0.1)/ 0.47 (0.1)/ 0.36 (0.1)	0.41 (0.11)/ 0.23 (0.06)/0.21 (0.06)	0.78/ 0.78/ 0.78	13.92/ 13.83/ 13.83	0.02/ 0.021/0.021
	linear	0.05 (0.17)/ *0.24 (0.16)/ 0.27 (0.16)*	0.24 (0.07)/ *0.11 (0.04)/ 0.05 (0.02)*	0.74/ *0.63/ 0.6*	11.1/ *6.76/ 6.01*	0.029/ *0.06/0.07*

We found that both pre- and postmating isolation increased with time since isolation of the two parental taxa. However, while premating isolation increased at a faster rate initially and then only slowly gained in strength over the following million years of divergence time (Fig. 2), single components of postmating incompatibility showed a gradual and consistent increase starting out low with fully viable hybrids between closely related species, which was then followed by a steady accumulation until complete inviability was reached after 4.4/8.5/18.4 my (Fig. 2 and 3). While premating isolation only increased by 9/4/2/% per million years divergence time (Table 1), fertilization failure accumulated at a rate of 24/11/5% (Table 2), and larval mortality (14-day mortality) at 29/13/6% per my. Total inviability (measured as the number of survivors after 180 days divided by the number of eggs that were laid initially) increased at a rate of 16/7/3 % per my. The maximum genetic distance to produce viable hybrids was ultimately determined by mortality before the free swimming stage. Partial viability (51 %) was observed between species that had been separated for 2.74/3.7/7.43 million years. Similar to previous studies (Coyne and Orr 1997; Price and Bouvier 2002; Mendelson 2003; Bolnick and Near 2005), the weighted node-averaging procedure used to improve the phylogenetic independence in our data set did not affect the major conclusions of our study, even though applying phylogenetic contrasts results in a reduction of sample size.

Our data suggest that the waiting time to complete intrinsic isolation between haplochromine cichlid species is much longer than the rates of speciation observed within individual lake radiations, contradicting the assumption of models with threshold-based incompatibility (Orr and Turelli 2001; Turelli and Moyle 2007), that the time to complete hybrid inviability equals the time since speciation. Our results suggest that the evolution of hybrid inviability plays little role in driving speciation in cichlids. Instead, other forces such as divergent ecological selection and disruptive sexual selection probably play the paramount role in the evolution of cichlid fish (Kornfield and Smith 2000; Seehausen 2000; Streelman and Danley 2003; Kocher 2004; Stelkens and Seehausen submitted). We conclude that extrinsic mechanisms are largely responsible for the maintenance of the immense cichlid diversity in the Great African Lakes, which renders this species system highly vulnerable to changes in the environment because reproductive isolation can break down under habitat disturbance (Seehausen et al. 1997).

Theoretical work predicts that in species with strong sexual selection, premating isolation between allopatric species can decay faster than postmating compatibilities, because even weak selection in allopatric sister populations can cause rapid changes in mate choice resulting in assortative mating (Fisher 1930; Lande 1981; West-Eberhard 1983). In agreement with this prediction, an experiment on darter fish (*Etheostoma*) showed that premating isolation between allopatric species accumulated at a faster rate than postzygotic compatibility (Mendelson 2003). Opposite to that, we here demonstrate a case of allopatric species pairs, each with a polygynous mating system and strong sexual dimorphism, where the decay of intrinsic, postmating compatibilities proceeds at a rate two- to three-fold faster than the decay of premating isolation. Hence, our results also do not support the theoretical prediction that pre- and postmating incompatibilities accumulate at similar rates in allopatry because genetic drift affects every trait type equally (Coyne and Orr 2004). The pattern we find may be explained by the fact that cichlid fish have a rather conserved courtship behaviour across lineages (McElroy and Kornfield 1990) which may be responsible for the rather weak mating barriers across large genetic distances we found. In fact, when comparing geographically close and distant populations of cichlids, stronger premating isolation is typically found between the sympatric pairs even though they are usually more closely related (Seehausen et al. 1998; Knight and Turner 2004; Plenderleith et al. 2005; Stelkens et al. 2008; Stelkens & Seehausen, submitted).

Our study reveals that instrinsic postmating incompatibility can be strongly stage-specific, suggesting that studies using a single estimate of hybrid inviability may lead to inaccurate conclusions about the relationship between hybrid fitness and genetic distance. Eggs that were successfully fertilized hatched at a rate unrelated to the genetic distance between the parental species. Similarly, fry that survived the first 14 days until the free swimming stage, lived to day 180 regardless of the divergence time between their parents. Our data suggest that those hybrids surviving the first two weeks of larval development are not impaired by genetic incompatibilities later in life (Fig. 3). It seems that the principal mechanisms responsible for hybrid viability operate during zygote formation and post-hatching larval development.

Because our study aimed to quantifying overall reproductive compatibility and isolation at various genetic distances we only have anecdotal observations of the specific factors limiting hybrid fitness during the larval stage. However, we observed an interesting deformity (Fig. 4) that occurred in numerous hybrid clutches between two and five days after hatching and before the free-swimming stage. It resembles a developmental mutation originally named *heartstrings* in zebrafish (Garrity et al. 2002), and more recently observed in centrarchid hybrids (Lopez-Fernandez and Bolnick 2008). While the syndrome allows the heart to develop normally during early embryonic stages, it causes a 'looping failure' in hatchlings and the heart adopts a string-like and elongate shape. In zebrafish the deformation is caused by a homozygous recessive mutation in the T-box family transcription factor tbx5, which can also lead to heart diseases in mice and humans (Garrity et al. 2002). Lopez-Fernandez and Bolnick (2008) suggested this may be the first candidate for a locus affected by Dobzhansky-Muller epistatic effects in vertebrates. However, identifying the genetic basis of reproductive isolation in our model system requires further investigation.
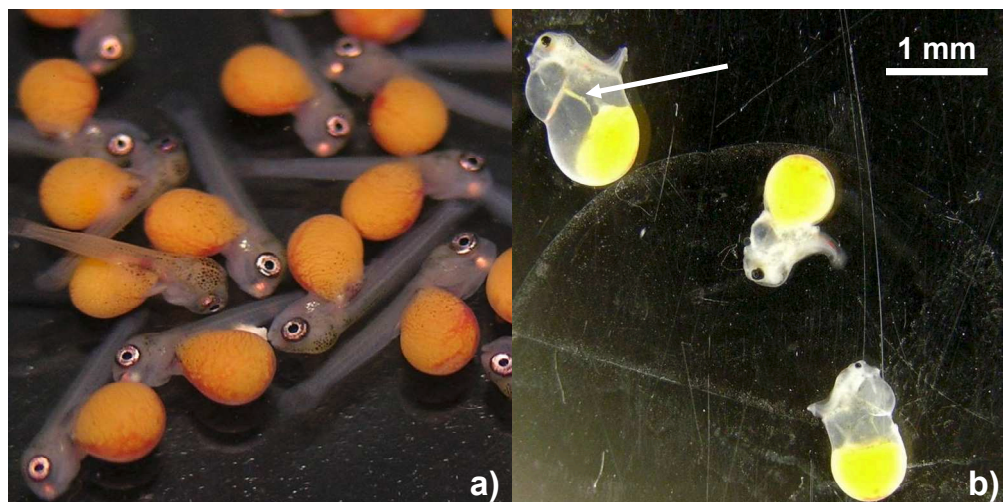
Fig. 4: a) Morphologically normal and b) deformed nine-day-old hybrid larvae. Scale bar is approximate. Arrow indicated the deformity that appears to correspond to the *heartstring* syndrome described for zebrafish and recently observed in sunfish hybrids. See discussion for references.

Dobzhanzky-Muller-incompatibilities are assumed to form the basis of most intrinsic hybrid inviabilities (Orr and Turelli 2001). They typically evolve as pleiotropic byproducts of genetic drift or natural selection after populations become isolated and are neutral or beneficial in their population of origin, but show negative epistasis when combined in a hybrid genome

(Turelli and Orr 2000). Because epistatic effects accumulate in a quadratic fashion in the Dobzhanzky-Muller model, incompatibility is predicted to increase slowly with genetic distance at first, but then with accelerating speed. Interestingly, there is very little evidence for this expected 'snowball effect' (Johnson 2006). Similar to data from other species groups studied so far (Coyne and Orr 1989, 1997; Turelli and Begun 1997; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002) our data do not support this pattern but instead reveal a linear decrease in compatibility over time. Gourbiere and Mallet suggest that - apart from potential sources of statistical noise such as the inconsistency of the speciation clock - few genes with major and variable effects and the concurrence of several different overlapping 'snowball' processes can blur the fit of the snowball function (Gourbiere and Mallet submitted).

To our knowledge, this is the first study on the decay of reproductive compatibilities that converts genetic distances into absolute evolutionary age using relaxed molecular clocks. Calibrating the time window for successful hybridization in the cichlid model system is particularly important because hybridization between distantly related lineages has been implicated in contributing to the exceptionally species-rich and rapid adaptive radiations of cichlid fish. The *hybrid swarm origin* hypothesis (Seehausen 2004) predicts hybridization to be common when populations invade new environments where - as a result of the enhanced genetic variation - hybrid populations may exhibit elevated rates of response to diversifying ecological selection allowing them to radiate faster than populations with a single ancestor, in which standing genetic variation becomes rapidly exhausted by the strong selection required for ecological adaptation. Our results confirm the mechanistic feasibility of the *hybrid swarm origin* hypothesis, demonstrating that the possibility to hybridize in cichlid fish does not cease for a long time after lineages have split (4.4/8.5/18.4 my). This time frame is consistent with the hypothesized hybridization events between the putative ancestors of two major cichlid radiations (Lake paleo-Makgadikgadi (Joyce et al. 2005) and Lake Victoria (Seehausen et al. 2003)) where the hybridizing lineages are thought to have been separated since ca. 10 my and ca. 8-15 my, respectively. Our finding, that hybrids can in fact show higher survival rates than their parental taxa, once they have survived certain embryonic and larval developmental stages, hints at heterosis-like effects on hybrid viability due to the overall increased heterozygosity in the F1 generation which may be another factor facilitating cichlid radiations with hybridization background. However, hybrid breakdown in generations succeeding the F1 can crucially contribute to the magnitude of reproductive isolation (Wu and Palopoli 1994). We are currently extending this experiment to F2 generation hybrids (Schmid, Stelkens & Seehausen, in preparation).

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SUPPLEMENTARY MATERIAL

Supplementary Table 1: NCBI Genbank accession numbers of D-loop sequences used for calculations of genetic distances. Asterisks indicate cases where no or insufficient sequences were available for the species used in the hybridization experiments. These were substituted for sequences of the most closely related species in Genbank (in brackets), which is justified by the absence of mitochondrial lineage sorting within the radiation of Lake Victoria cichlids and the Malawi Mbuna.

species	Genbank accession numbers
* Pundamilia pundamilia/nyererei	AF213528, AF213544, AF213545, AY930005, AY930006 (Neochromis nigricans)
* Neochromis omnicaeruleus	AF213540, AF213539, AF213525 (Paralabidochromis chilotes)
* Paralabidochromis rockkribensis/chilotes	AF213546, AF213548, AF213547, AF213529 (Paralabidochromis plagiodon)
* Metriclima estherae / zebra	AY930025 (Metriaclima. zebra), AF213620, AY911810, AY911811, AY911812 (Metriaclima callanois)
Astatotilapia calliptera	AF298938, AY911722, AY929977, AF298940, AF298939, AF298941, AY911723
Astatotilapia burtoni	AY929999, AF298905, AY929955, AF298906, AY930000, AY930001, AF298904
Protomelas taeniolatus	AF298963, AY913942, EF6475464
Pseudocrenilabrus philander	AY913860, AY913853, AY913859
Pharyngochromis acuticeps	AY913913, AY913848, AY913845, AY913882, AY913912, AY913846
Thoracochromis buysi	AY913883, AY913851
Thoracochromis demeusii	AY913857
Ctenochromis horei	AY301953, AY301952, AY929987
Sargchromis sp.	AY913895, AY913904, AY913905, AY913894, AY913893
Oreochromis tanganicae (outgroup)	AY929940
Bathybates sp (outgroup)	BSU12556

Supplementary Table 2: Twenty-six different interspecific hybrid crosses between 16 species of haplochromine cichlids included in this study with pairwise genetic distances (uncorrected p-distance calculated from mt D-loop sequences), divergence times (in millions of years calculated with an internally calibrated linear clock, and two different relaxed non-linear clocks), and the geographical status of each species pair (sym = sympatric, allo = allopatric). Sex-reversed crosses of the same type are indicated by 'a' and 'b'. Numbers in the last four columns are the number of clutches per cross type available to measure four different elements of postmating isolation. Numbers in brackets are the number of individuals per clutch that survived until 180 days. Shaded rows indicate cross types for which only premating isolation data was obtained.

cross type	male parent	female parent	geography	gen. dis-tance	divergence time internal clock	divergence time fossil record	divergence time Gond-wana break up	n spaw-nings for pre-mating isolation	n clutches for fertili-zation rate	n clutches for hatching rate	n clutches for 14 day survival rate	n clutches for 180 day survival rate
1	*Neochromis omnicaeruleus*	*Pundamilia pundamilia*	sym	0.007	0.35	0.104	0.135	7	4	4	4	4 (36,37,24, 30)
2	*Paralobidochromis chilotes*	*Pundamilia nyererei*	sym	0.007	0.35	0.104	0.135	8	2	2	2	2 (27,27)
3	*Paralobidochromis rockkribensis*	*Pundamilia pundamilia*	sym	0.007	0.35	0.104	0.135	4	2	2	2	2 (53,29)
4a	*Astatotilapia calliptera*	*Metriaclima estherae*	allo	0.0188	0.93	0.58	0.919	5	5	5	5	5 (3,11,9,12,12)
4b	*Metriaclima estherae*	*Astatotilapia calliptera*	allo	0.0188	0.93	0.58	0.919	3	3	3	3	3 (12,12,21)
5a	*Protomelas taeniolatus*	*Astatotilapia calliptera*	allo	0.0241	1.19	0.891	1.485	4	4	3	3	2 (22,42)
5b	*Astatotilapia calliptera*	*Protomelas taeniolatus*	allo	0.0241	1.19	0.891	1.485	2	2	-	-	
6a	*Astatotilapia burtoni*	*Astatotilapia calliptera*	allo	0.0408	2.02	2.226	4.117	15	14	11	11	11 (6,2,20,34,1,22,37, 64,20,33)
6b	*Astatotilapia calliptera*	*Astatotilapia. burtoni*	allo	0.0408	2.02	2.226	4.117	2	2	-	-	
7	*Astatotilapia burtoni*	*Metricalima estherae*	allo	0.0483	2.39	2.981	5.7	2	1	-	-	
8a	*Astatotilapia burtoni*	*Pundamilia pundamilia*	allo	0.0543	2.69	3.665	7.176	9	6	4	4	
8b	*Pundamilia pundamilia*	*Astatotilapia burtoni*	allo	0.0543	2.69	3.665	7.176	8	8	-	-	
9a	*Metriaclima zebra*	*Pundamilia pundamilia*	allo	0.0552	2.73	3.766	7.397	12	12	10	10	
9b	*Pundamilia pundamilia*	*Metriaclima zebra*	allo	0.0552	2.73	3.766	7.397	4	4	-	-	
10a	*Metriaclima estherae*	*Pundamilia pundamilia*	allo	0.0552	2.73	3.766	7.397	3	-	-	-	
10b	*Pundamilia pundamilia*	*Metriaclima estherae*	allo	0.0552	2.73	3.766	7.397	4	-	-	-	
11	*Neochromis omnicaeruleus*	*Metriaclima estherae*	allo	0.0552	2.73	3.766	7.397	1	-	-	-	
12a	*Astatotilapia calliptera*	*Pundamilia nyererei*	allo	0.0553	2.74	3.779	7.426	14	14	9	9	
12b	*Pundamilia nyererei*	*Astatotilapia. calliptera*	allo	0.0553	2.74	3.779	7.426	14	14	11	10	10 (21,9,34,31,27,24, 30,20,6,15)
13	*Astatotilapia calliptera*	*Pundamilia pundamilia*	allo	0.0553	2.74	3.779	7.426	2	-	-	-	
14	*Ctenochromis horei*	*Pundamilia pundamilia*	allo	0.0752	3.72	6.452	13.48	0	-	-	-	
15	*Pharyngochromis acuticeps*	*Astatotilapia calliptera*	allo	0.0808	4.00	7.311	15.496	4	-	-	-	
16	*Thoracochromis buysi*	*Pundamilia pundamilia*	allo	0.0854	4.22	8.05	17.253	0	-	-	-	
17	*Thoracochromis buysi*	*Pundamilia nyererei*	allo	0.0854	4.22	8.05	17.253	2	-	-	-	

18a	*Thoracochromis demeusii*	*Paralobidochromis rockkribensis*	allo	0.0854	4.22	8.05	17.253	1	-	-	-
18b	*Paralobidochromis rockkribensis*	*Thoracochromis demeusii*	allo	0.0854	4.22	8.05	17.253	0	-	-	-
19	*Astatotilapia calliptera*	*Pseudocrenilabrus philander*	allo	0.0855	4.23	8.066	17.293	0	-	-	-
20a	*Sargochromis sp.*	*Pundamilia nyererei*	allo	0.0883	4.37	8.531	18.408	8	4	3	3
20b	*Pundamilia nyererei*	*Sargochromis sp.*	allo	0.0883	4.37	8.531	18.408	1	1	-	-
21a	*Sargochromis sp*	*Pundamilia pundamilia*	allo	0.0883	4.37	8.531	18.408	0	-	-	-
21b	*Pundamilia pundamilia*	*Sargochromis sp.*	allo	0.0883	4.37	8.531	18.408	1	1	-	-
22	*Pharyngochromis acuticeps*	*Pundamilia nyererei*	allo	0.0912	4.51	9.025	19.599	1	1	-	-
23	*Pharyngochromis acuticeps*	*Pundamilia pundamilia*	allo	0.0912	4.51	9.025	19.599	1	1	-	-
24a	*Pundamilia nyererei*	*Pseudocrenilabrus philander*	allo	0.0943	4.66	9.565	20.912	2	-	-	-
24b	*Pseudocrenilabrus philander*	*Pundamilia nyererei*	allo	0.0943	4.66	9.565	20.912	4	-	-	-
25	*Pundamilia pundamilia*	*Pseudocrenilabrus philander*	allo	0.0943	4.66	9.565	20.912	0	-	-	-
26	*Pharyngochromis acuticeps*	*Pseudocrenilabrus philander*	allo	0.0968	4.79	10.01	22.001	0	-	-	-

Supplementary Table 3: Proportion of spawned females per experimental observation unit (aquarium) with genetic distance of the cross type and the duration of the trial in n of days.

aquarium	genetic distance	trial duration (days)	proportion of spawned females
1	0	83	0.43
2	0	119	0.67
3	0	162	0.2
4	0	401	0
5	0	311	1
6	0	182	0.4
7	0.029	131	0.38
8	0.029	43	0.38
9	0.034	64	0.22
10	0.034	64	0.08
11	0.034	126	0.67
12	0.047	133	0.35
13	0.047	46	0.06
14	0.047	480	0.29
15	0.047	231	0.2
16	0.047	357	0.18
17	0.047	206	0.29
18	0.047	169	0.5
19	0.052	41	0.07
20	0.052	154	0.7
21	0.052	593	0.25
22	0.055	64	0.08
23	0.055	64	0
24	0.055	64	0.11
25	0.066	132	0.86
26	0.066	256	0.22
27	0.066	153	1
28	0.069	108	0.17
29	0.069	286	0.83
30	0.069	110	0.08
31	0.069	231	0.05
32	0.069	142	0.1
33	0.069	142	0.2
34	0.094	102	0.06
35	0.094	126	0
36	0.094	89	0.13
37	0.094	232	0
38	0.094	163	0
39	0.094	196	0.2
40	0.094	197	0.38
41	0.116	108	0.05
42	0.116	256	0.2
43	0.116	257	0
44	0.116	126	0
45	0.116	215	0.57
46	0.116	293	0
47	0.116	293	0.22
48	0.116	293	0
49	0.116	124	0
50	0.116	257	0.4
51	0.116	24	0
52	0.116	297	0
53	0.116	149	0.33
54	0.116	480	1
55	0.116	254	0
56	0.116	444	0
57	0.116	107	0.08
58	0.116	124	0.2

Chapter 5

A new African Great Lake cichlid radiation emerged from five distantly related lineages through genetic exchange and competition

Rike B. Stelkens[1, 2, 3], Cyprian Katongo[5], Domino A. Joyce[4], Ole Seehausen[1, 2] *

1. Division of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, Baltzerstr. 6, CH-3012 Bern, Switzerland
2. Department of Fish Ecology and Evolution, Centre of Ecology, Evolution and Biogeochemistry (CEEB), Eawag Swiss Federal Institute of Aquatic Science and Technology, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland
3. Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland
4. Molecular & Evolutionary Ecology Group, Department of Biological Sciences, University of Hull, Hull HU6 7RX, U.K
5. Department of Biological Sciences, University of Zambia at Lusaka, Zambia

* Corresponding author: email: ole.seehausen@eawag.ch; tel: +41 41 349 2121; fax: +41 41 349 2168

ABSTRACT

A major challenge in adaptive radiation research is to identify the roles of extrinsic environmental versus intrinsic organismal in controlling rates of diversification. Here, we demonstrate a case in which multiple related colonizing lineages undergo adaptive radiation simultaneously in the same new environment, co-evolving through genetic exchange and ecological interaction. This study describes, for the first time, the diversity of haplochromine species in an understudied African Great Lake, representing a major adaptive cichlid radiation that had gone completely unnoticed to date. We identified > 40 phenotypically distinct taxa, varying in size, body shape, jaw morphology, male breeding colouration, and habitat use in Lake Mweru (Zambia/DRC). Several of the taxa resemble well known lineages from outside the area. Others represent completely new, endemic types. A mitochondrial genealogy reveals that at least eight phylogenetically distinct lineages of haplochromine cichlids must have independently colonized Lake Mweru which resulted in four different sub-radiations in the genera *Sargochromis*, "large tooth" *Serranochromis*, "small tooth" *Serranochromis*, and *Pseudocrenilabrus*. Each of these new radiations contains at least five to 15 phenotypically distinct taxa. Strikingly, we found traces for hybridization in all four sub-radiations, witnessed by mitochondrial haplotype capture between the deeply divergent lineages. Nuclear genomic monophyly of the three serranochromine sub-radiations suggests that hybridization must have been ancient, probably before the species radiations started. Dating the onset of each sub-radiation using three different (relaxed) molecular clocks indicates that all four radiations started at a similar point in time between 0.18-0.94 MY ago. Analysis of morphological disparity shows that the sub-radiations are complementary to each other in morphospace indicative of competition for resources constraining the direction and volume of phenotypic evolution within radiations. Together, Lake Mweru's large phenotypic diversity resembles that of other Great African Lakes and harbours ecologically relevant shape variation comparable in magnitude to the classic examples of cichlid adaptive radiations. These data are the strongest yet available evidence that ancient hybridization between distant lineages is associated with rapid diversification of cichlid species, and that competition between major lineages causes co-evolutionary patterns in the ecomorphological diversification of each sub-radiation.

KEYWORDS: adaptive radiation, cichlids, coevolution, cyto-nuclear discordance, genetic variation, hybridization, speciation

INTRODUCTION

The adaptive radiations of haplochromine cichlid fish in the Great African Lakes are the largest and fastest radiations of animals known. Hundreds of species have evolved in Lakes Victoria, Malawi, Tanganyika and Edward (Genner et al. 2004; Kocher 2004), sometimes in astonishingly short periods of time (Seehausen 2002; Genner et al. 2007; Stager and Johnson 2008). Explaining the extraordinarily high rates of speciation and morphological evolution, and the unusually large numbers of closely related sympatric species is no minor challenge to evolutionary biologists. Equally unexplained is why these incredible radiations happened in some African Great lakes, but not in others, despite the presence of haplochromine cichlids in all of them. Various hypotheses have been proposed, but all fall short of explaining the patterns of differential diversification (Seehausen 2006). One often cited hypothesis is evolutionary release from competition in supposedly isolated lakes (Fryer and Iles 1972). This hypothesis postulates that the presence of a diverse array of other fish constrains the diversification of cichlids. A more recent hypothesis is that rapid radiations occur when several distantly related cichlid species colonized the same lake and hybridized (Seehausen 2004). Although long thought of as monophyletic, recent data suggest the possibility of such hybrid swarm origins for some cichlid fish radiations (Seehausen et al. 2003; Joyce et al. 2005).

Lake Mweru, in the upper Congo (Luapula) drainage in Zambia/Democratic Republic of Congo, is a large lake that would supposedly be relatively isolated (against upstream migration from the Congo River by water falls), and contains several distantly related riverine cichlid species, yet was thought to have no radiation. The same is true for nearby Lake Bangweulu which is slightly smaller, and is further upstream in the same drainage. We visited these lakes for a comparative investigation of their cichlid populations. In Lake Bangweulu we found the six non-endemic haplochromine species decribed from the lake, plus possibly one new species. We found indeed no evidence for a radiation. In Lake Mweru, on the other hand, we found a large diversity of undescribed haplochromine species, representing a major radiation that had remained completely unrecognized. Several of the taxa resembled well known lineages from outside the area, such as members of the genera *Serranochromis* and *Sargochromis*. Others represented completely new phenotypes not known from anywhere else. Among these was a larger number of diverse species evidently closely related to the widespread but elsewhere species-poor Panafrican genus *Pseudocrenilabrus*. Across all groups, we identified more than 40 phenotypically distinct, putative species with up to 15 at single collection sites, varying in, size (from 3 to 30cm), body shape, jaw morphology, male breeding colouration, and habitat use. Geological evidence suggests that Lake Mweru was unlikely to be an old lake. It is merely 27m deep, and would probably have been dry by the end of the Pleistocene when the 80m deep Lake Victoria was dry (Johnson et al. 1996; Stager and Johnson 2008) and the nearby Lake Tanganyika had fallen by 350m (Gasse et al. 1989; Scholz et al. 2003).

Here we aim at reconstructing the evolutionary dynamics that led to this cichlid radiation. Specifically, we recover the phylogenetic complexity of a young and large haplochromine radiation, the ecological and genetic relationships between its seeding lineages and the emergent species, and the temporal patterns of radiation. We are particularly interested in understanding how multiple distantly related but ecologically similar colonizing lineages interact during adaptive radiation. We sequenced the mitochondrial control region (D-loop) of 228 individuals from Lakes Mweru and Bangweulu, and combined these data with published sequences of the closely related Makgadikgadi radiation (n = 98) and representatives of all other major African radiations (Lakes Victoria, Malawi, Tanganyika) and riverine haplochromines. We dated the onset of the Mweru radiation within the mitochondrial genealogy with three different molecular clocks. We reconstructed a nuclear phylogeny of the radiation, using 1331 genomic AFLP loci genotyped in a strategically

chosen subset of samples. To assess the magnitude of eco-morphological variation present in the Lake Mweru radiation, and to characterize its distribution, we measured 13 linear morphometric distances that reflect ecologically relevant shape variation. We compared the eco-morphological variation with that found in the classical radiations of Lakes Victoria, Malawi and palaeo-Lake Makgadikgadi.

Our mitochondrial genealogy revealed that at least eight phylogenetically distinct lineages of haplochromine cichlids must have independently colonized Lake Mweru. The endemic species diversity, however, radiated very recently and quasi simultaneously in four of the haplotype lineages. The recency of the radiation is comparable to that of the Lake Victoria cichlids. Yet, it involved lineages that, despite being present, are not known to have radiated in other African lakes. In all four lineages we found evidence for ancient hybridization with very distantly related lineages, witnessed by mitochondrial haplotype capture between lineages, that had diverged for 3.11/4.72/9.51 MY, 2.90/4.20/8.34 MY, 2.52/3.27/6.32 MY (among "large tooth *Serranochromis,* "small tooth" *Serranochromis,* and *Sargochromis*), and 1.70/1.65/2.94 MY (*Pseudocrenilabrus*) divergence time (estimates depending on the molecular clock used), resulting in three *Serranochromis/Sargochromis* radiations and one *Pseudocrenilabrus* radiation. The eco-morphological diversity of these sub-radiations combined, resembles that observed in the classical radiations of Lakes Victoria and Malawi. The four Mweru sub-radiations filled almost completely non-overlapping sections of the overall radiation morphospace, suggesting that their presence impinges upon the morphological diversification of others.

We conclude that a major adaptive radiation of cichlids had gone unrecognized in a poorly studied African Great lake. Our genetic and phenotypic data provide new insights into the evolutionary dynamics of adaptive radiation in cichlid fish, specifically into two much debated, but not yet well resolved issues, the role of hybridization and that of competition between distant relatives: 1) Hybridization occurred even between distantly related colonizing lineages, and led to apparent nuclear genomic monophyly. 2) The presence of other haplochromine lineages appears to have constrained adaptive radiation, generating distinct patterns of co-radiation between major clades. This is the strongest yet available evidence that competition can indeed constrain cichlid adaptive radiation. At the same time this is the strongest yet available evidence that adaptive radiation of cichlids is associated with hybridization even between distantly related colonizing lineages.

METHODS

Fieldwork
Between September and October 2005 we sampled eleven locations along the southern and eastern coasts of Lake Mweru (Fig. 1), including extensive shallow, sandy beaches in the south, a massive rocky outcrop on Kilwa Island in the south-west, steeply sloping, wave exposed, sandy beaches and rocky boulder shores in the north-east, and offshore open waters in the south, south-west and north-east. We also sampled two sites in the large lagoon network directly south of Lake Mweru. In Lake Bangweulu, we sampled five locations (Fig. 2), including white sand beaches, very large water lily beds, and extensive reed swamps. Catching methods included monofilament gill nets and beach seines operated by teams of professional fishermen. Other fish were obtained from angling youth at rocky shores, from offshore commercial gillnet fisheries (fishing methods per site are indicated in Fig. 1 and 2), and from one large commercial landing site in each lake. We collected a total of 404 specimens from Lake Bangweulu and 767 specimen from Lake Mweru, which were first preserved in formalin and then transferred to 75% ethanol in the laboratory. Fish are held in the collection of the University of Lusaka, Zambia, and at EAWAG, Switzerland.

Taxon sampling and DNA extraction

We sequenced the mitochondrial control region (D-loop) of 228 individuals, 51 from Lake Bangweulu, and 177 from Lake Mweru and reanalyzed 98 GenBank accessions (AY913844–AY913942) from a previous study of the mitochondrial phylogeography of South East African cichlids (Joyce *et al.* 2005), as well as 60 GenBank accessions of haplochromine cichlids from all other African Great lake species flocks, and East African rivers (a detailed list of all specimens including sampling localities, and Genbank accession numbers is provided as Supplementary Table 3). Fin clips were preserved in 95% ethanol. DNA was extracted using proteinase K digestion and Promega Wizard DNA extraction kit (Promega) following the manufacturer's protocol. All samples were standardized to a DNA concentration of 50 ng/μl.

mtDNA sequence analysis

PCR, purification of PCR products, and sequencing followed the protocol described in Joyce et al. (2005). DNA fragments were amplified using DTCS quickstart (Beckman Coulter) according to the manufacturer's instructions adding 1 M betaine to the sequencing reaction. Sequences were resolved using a Beckman capillary sequencer. The D-loop sequence of the mitochondrial control region was amplified using forward primer Dloopint 5'–AGCCCACCATCAGTTGATT-3' and reverse primer HapDloop 5'-GGTTTGCAGGAGTCTTAGAG-3'. Alignment of sequences was done in ClustalX, using pairwise alignment (Thompson *et al.* 1997) and adjusted by eye. The alignment was comprised of 386 sequences, each 706 bp long (to be submitted to Genbank). Modeltest3.7 (Posada and Crandall 1998) was used to estimate likelihood parameters. The model best fitted to our data was the general time reversible substitution model with among site rate heterogeneity following a gamma plus invariant sites distribution (GTR + I + Γ, shape parameter 0.6416, proportion of invariable sites 0.1617, empirical base frequencies: A = 0.3186, C = 0.1902, G = 0.1455, T = 0.3457). A maximum likelihood tree was built in PhyML3.0 (Guindon and Gascuel 2003) using the parameters from Modeltest and 100 bootstrap pseudoreplicates.

To compare the timing of the four radiations in Lake Mweru, we calibrated the major nodes in our phylogeny with time estimates obtained from Genner et al. (2007). Genner et al. developed two relaxed molecular clocks, the first based on a combination of cichlid fossil records and recent geological events, and the second based on a combination of the fragmentation of Gondwanaland and the same recent geological events. Both demonstrate that rates of molecular evolution of the cichlid D-loop region are time dependent, with a rapid decline until a baseline substitution rate after approximately one to two million years divergence time is reached. In addition to that, we also applied a linear clock that was internally calibrated using the age of Lake Malawi (Sturmbauer et al. 2001).

Kalobwa (offshore gillnets)

Kalobwa (rocky boulder shore)

Kalobwa (beach seine over sand/mud)

Kalungwishi River
(rocky rapids just above river mouth)

Kabuta (beach seine over sand/mud)

Kabuta (rocky boulder shore, angling youth)

Kashikishi (beach seine over sand)

Nchelenge (regional fish landing site)

Kilwa/Isokwe (offshore gillnets)

Kilwa Island (rocky outcrops)

Isokwe Island (beach seine over sand,
gillnets along macrophyte belt)

Chief's Pond (gillnets)

Pembe Lagoon (gillnets)

Fig. 1: Sampling region Lake Mweru (Zambia/DRC) with collection sites and fishing methods

Mulongwe (traps, angling women)

Samfya (Fish Market and beach landing site)

Kapapa (beach seine, gillnets)

Mofya (large waterlilly beds, gillnets)
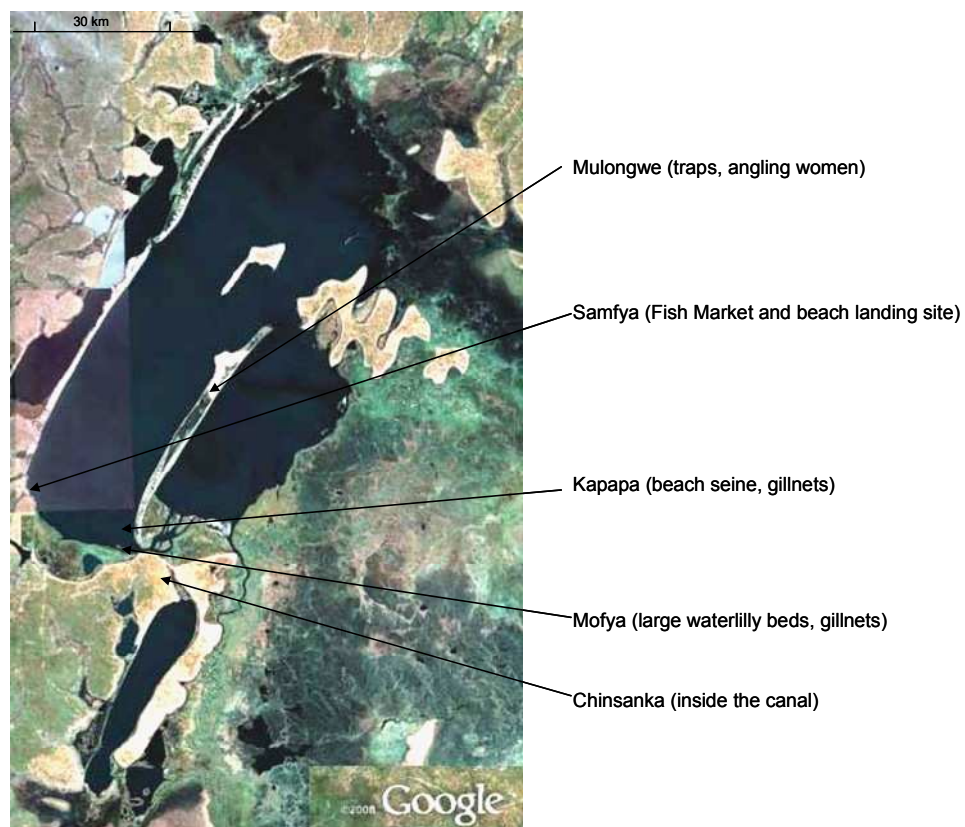
Chinsanka (inside the canal)

Fig. 2: Sampling region Lake Bangweulu (Zambia) with collection sites and fishing methods

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AFLP analysis

AFLP genotypes were obtained from a subset of the Lake Mweru individuals in the mtDNA tree. This subset contained five individuals showing a very conspicuous mismatch between their mitochondrial haplotype clade affiliation and their phenotypic affiliation. For comparison we also included individuals where phenotype and haplotype clade were in agreement. The distantly related species *Thoracochromis demeusii* (from the lower Congo River) and *Metriaclima estherae* (endemic to Lake Malawi) were used as outgroups. We used the following protocol. Restriction digestion and adaptor ligation were carried out using 1.1 μl T4 ligase buffer (1 x), 1.1 μl NaCl (50 mM), 0.55 μl BSA (50 μg/ml), 0.02 μl *Mse*I (0.09 units/μl, New England BioLabs), 0.05 μl *Eco*RI (0.45 units/μl, New England BioLabs), 1 μl *Mse*I-adaptor (50 μM), 1 μl *Eco*RI-adaptor (5 μM), 0.06 μl T4 DNA ligase (5.56 units/μl) and 0.62 μl HPLC water, and incubated for two hours at 37°C. Of the restriction-ligation product, 3 μl were used in the preselective amplification reaction, also containing 1 μl of each of the *Mse*I and *Eco*RI preselective primers (0.5 μM), 2 μl NH_4 reaction buffer (1 x), 1 μl $MgCl_2$ (2.5 mM), 2 μl dNTPs (200 μM), and 0.15 μl *Taq* DNA polymerase (0.04 units/μl, Bioline). Preselective primers were identical to the adaptor primer sequence with a single nucleotide added at the 3'- end (*Mse*I-pre: C, *Eco*RI-pre: A).

We conducted a preselective amplification with one selective base on each primer (Mse I-C and Eco RI-A). The preselective PCR used the following temperature profile: 2 min at 72°C, 20 cylces of 20 sec at 94°C, 30 sec at 56°C, 2 min at 72°C, then holding for 30 min at 60°C. This was followed by 12 different selective amplifications, using 12 different combinations of primers with an additional two-base extension. PCR products were diluted 1:10 for selective amplification and we used the following 12 primer combinations: *Mse*I-CTT/*Eco*RI-AAG, *Mse*I-CTT/*Eco*RI-ACA, *Mse*I-CTT/*Eco*RI-AGC, *Mse*I-CTC/*Eco*RI-AAG, *Mse*I-CTC/*Eco*RI-ACA, *Mse*I-CTC/*Eco*RI-AGC, *Mse*I-CTA/*Eco*RI-AGG, *Mse*I-CTA/*Eco*RI-ATC, *Mse*I-CTA/*Eco*RI-ATT, *Mse*I-CAT/*Eco*RI-AAG, *Mse*I-CAT/*Eco*RI-ACA, *Mse*I-CAT/*Eco*RI-AGC. Selective PCR contained 1.5 μl diluted preselective PCR product, 0.4 μl $MgCl_2$ (2.5 mM), 1 μl NH_4 reaction buffer (1 x), 1 μl dNTPs (200 μM), 0.1 μl *Taq* DNA polymerase (0.04 units/μl), 2.5 μl selective *Mse*I primer (0.25 μM), 0.5 μl selective *Eco*RI primer (0.05 μM), and 3 μl HPLC water. Selective primers were labelled with fluorescent dyes IR700 (Microsynth) and Alexa (Ax647, Ax700, Ax750, Invitrogen). The temperature profile for the selective PCR was: 2 min at 94°C followed by 10 cycles with 20 sec at 94°C, 30 sec at annealing temperature, decreasing in each cycle by 1°C from 66°C to 56°C, and 2 min at 72°C. The PCR continued for 20 cycles with 20 sec at 94°C, 30 sec at 56°C, and 2 min at 72°C, followed by a holding step at 60°C for 30 min. All amplifications were performed on a Techne TC-512 (Barloworld Scientific). Selective amplification products were visualized on a Beckman capillary sequencer with an internal size standard (Beckman Coulter).

We analyzed the raw fragment data on a CEQ8000 Genetic Analysis System sequencer (Beckman Coulter) using automatic scoring considering fragments within a size range of 60-400 bp. Five individuals (25% of all samples) were subjected to the same procedure twice (from DNA extraction, through restriction to selective amplification and data scoring) to evaluate reproducibility. The error rate per individual was calculated as the ratio between the observed number of differences (mismatches) and the total number of comparisons (number of fragments scored) (Pompanon *et al.* 2005). The average mismatch across the repeated individuals was 5.4%, hence repeatability was 94.6% for all 12 primer pair combinations. Scoring peaks by eye resulted in a higher mismatch between repeated samples. We hence decided to use only automated scoring, using the Beckman software for fragment analysis. We obtained a total of polymorphic 1331 fragments.

To build a tree from the AFLP data, we used the distance based Fitch-Margoliash method as implemented in Fitch (Phylip3.67), which maximizes the fit of the observed pairwise distances to a tree by minimizing the squared deviation of all possible observed

distances relative to all possible path lengths on the tree (Felsenstein 1997). We used 1000 bootstrap pseudoreplicates, global and local rearrangements, and 100 different randomly jumbled input sequences. For comparison with a mitochondrial genealogy of the subset of individuals genotyped at AFLP loci, we used PAUP*4.0b10 (Swafford 2001) to construct a neighbour joining tree of the mitochondrial haplotypes with 1000 bootstrap pseudoreplicates using parameters from Modeltest3.7 (GTR + I + Γ).
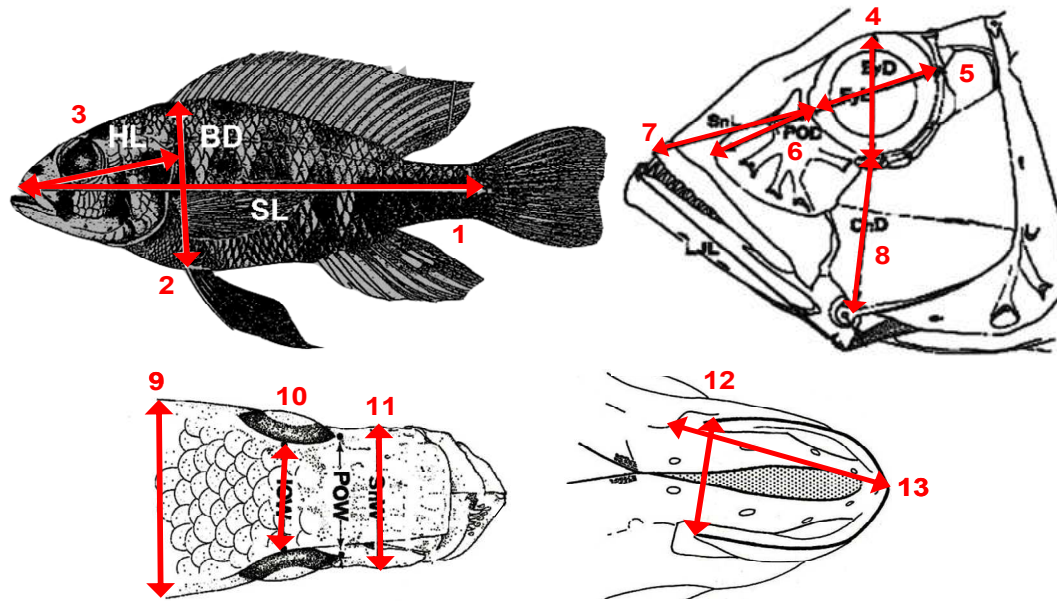

Fig. 3: Schematic drawing of the 13 morphological distances measured, indicated by the red arrows (modified from Barel et al. 1977). 1 = standard length (SL), 2 = body depth (BD), 3 = head length (HL), 4 = eye depth (EyD), 5 = eye length (EyL), 6 = preorbital depth (PoD), 7 = snout length (SnL), 8 = cheek depth (ChD), 9 = head width (HW), 10 = interorbital width (IoW), 11 = snout width (SnW), 12 = lower jaw width (LjW), and 13 = lower jaw length (LjL).

Phenotypic analysis

To characterize the ecological and phenotypic diversity in the new cichlid radiation of Lake Mweru, and its partitioning between lineages and between putative species within lineages, we phenotyped 192 individuals (35 *Sargochromis*, 37 large tooth *Serranochromis,* 40 small tooth *Serranochromis*, 80 *Pseudocrenilabrus*) for 13 standard morphometric distances, the combination of which is powerful to detect even fine eco-morphological differences between species (Barel et al. 1977; Seehausen et al. 1999). We measured with digital callipers to the nearest 0.01 millimetre (a detailed list of the species with collection sites is provided in Supplementary Table 4). We used at least 3 and maximally 15 individuals per putative species for all Lake Mweru species. 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) (Fig. 3). Trait variables were log-transformed to homogenize variance. Normal distributions were confirmed with Shapiro-Wilk tests.

There were significant differences in body size between species within each of the radiations (one-way ANOVA: *Sargochromis* (clade I): $F_{6,34} = 18.55$, $p < 0.001$; large tooth *Serranochromis* (clade III): $F_{3,36} = 79.69$, $p < 0.001$; small tooth *Serranochromis* (clade IV):

$F_{5,39} = 22.69$, p < 0.001; *Pseudocrenilabrus* (clade VII): $F_{12,79} = 6.14$, p < 0.001). Because most distances were significantly correlated to body size, we used a method that corrects for size differences between species (Hendry *et al.* 2002). Linear regressions were calculated within species for each of the distances against either standard length (BD, HL) or head length (the remaining nine distances). The species and distance-specific slopes (**b**) resulting from these regressions 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**) across all species using the expression $\mathbf{M_{std} = M_o (L_o/L_x)^b}$.

Adjusted distances were then entered into a principal component analysis (PCA) and the first three principal components were plotted for visualisation of the morphospace occupied by each species. This analysis was done separately for all four radiations. To ask if putative species within mtDNA haplotype clades were phenotypically differentiated, one-way ANOVAs were calculated on the first three principle components with putative species as factor, for each clade separately. Tukey-Kramer tests were applied for posthoc pairwise comparisons between species within clades.

We also compared the total morphological diversity of the Lake Mweru haplochromine radiations to the classical African Great Lake radiations of haplochromines using existing data of species from Lake Victoria (LV), Lake Malawi (LM), Lake palaeo-Makgadikgadi (LMkg) (Joyce et al. 2005). Because the data for some of the other radiations were only available as ratios of some of the distances described above (HL/SL, BD/SL, SnL/HL, LjL/LjW, ChD/HL, IoW/HL, PoD/HL, EyL/HL), the cross-lake comparative analysis was restricted to this subset of distances. In total, our data set contained 13 putative species of the *Sargochromis* clade, eight of the large tooth *Serranochromis,* 13 of the small tooth *Serranochromis*, and 24 of the *Pseudocrenilabrus* clade from Lake Mweru, 27 Lake Victoria species, 28 Lake Malawi species, 26 palaeo-lake Makgadikgadi species. We also included haplochromines from other East and North African lakes and rivers (11 species), and three *Serranochromis* species from Lake Mweru that are not part of the endemic radiations (*S. robustus, S. thumbergi, S. angusticeps*). Distances for each putative species of Lake Mweru were averages of at least three individuals. Principal component analysis was used to extract the major axes of variation. PC2 and PC3 were plotted against PC1 for visualization of the morphospace covered by each radiation. To test for morphological disparity between the four species rich clades within Lake Mweru, we used ANOVA with PC1, PC2 and PC3 as dependent variables and Lake Mweru clades as factors. We tested for pairwise comparisons between radiations using Tukey-Kramer tests.

RESULTS
Taxonomic diversity
The specimens from Lake Bangweulu could be readily identified as six described haplochromine species, five of which are widely distributed in the Zambezi system (*Pseudocrenilabrus philander*, *Serranochromis robustus*, *S. thumbergi*, *S. angusticeps*, *S. altus*), and one restricted to the Luapula drainage system (*Sargochromis cf. mellandi*). Some individuals of *Serranochromis* were intermediate in phenotype between the closely related species *S. angusticeps* and *S. altus* (Plate 1, Supplementary Material). These may represent hybrids or a third, undescribed species. Nuptial males of *Sargochromis cf. mellandi* had a conspicuously smoky-grey underside of head and anterior body. We refer to them as *S. "smoky face mellandi"* to distinguish these from the fish of Lake Mweru.

Very few of the specimens collected in Lake Mweru could be readily identified as species known from outside the lake, or for that matter as described species at all. These were *Serranochromis robustus* and *S. angusticeps*, both widely distributed in the Zambezi River system and beyond. The others represented an unexpected array of diverse phenotypes,

varying in size, form and colour. They fell into five major groups defined by a few key morphological features. 1) The strictly Congolese genus *Orthochromis*, defined by very elongate but cylindrical bodies and absence of any anal fin markings. 2) Fish resembling the genus *Pseudocrenilabrus* by virtue of showing a red spot on the trailing edge of the anal fin of males. This group included a species described as *Thoracochromis moeruensis* and several similar forms. 3) The genus *Sargochromis*, defined by many distinct ocelly ("egg spots") on the anal fin of males, relatively deep bodies and generalized morphology of the trophic apparatus. 4.) The genus *Serranochromis*, defined by many distinct ocelly on the anal fin of males, elongate bodies, and a distinctly predatory head morphology and large canine teeth (we refer to them as "large tooth *Serranochromis*"). 5) A group of *Serranochromis*-like fish with very small teeth (we refer to them as "small tooth *Serranochromis*") and variable body shapes, bridging between *Sargochromis* and large tooth *Serranochromis* (for illustration of all groups see Plate 1, Supplementary Material).

Mitochondrial phylogeography of Lake Mweru haplochromines

Our mitochondrial D-loop tree (Fig. 4) recovered all known major clades of haplochromine cichlids (Joyce et al. 2005; Salzburger et al. 2005). All serranochromine cichlids together made a well supported clade. A number of Congolese haplochromines, including *Thoracochromis demeusii*, *Orthochromis polyacanthus*, and one of our Lake Mweru *Orthochromis* (*sp.* "*red fin*") were the sister group to the serranochromines (83% bootstrap support). All *Pseudocrenilabrus*-like fish together (including the *Thoracochromis moeruensis* complex) also formed a well supported clade, and the other two of our Lake Mweru *Orthochromis* (*O. kalungwishiensis* from the Kalungwishi River, and *O. sp. "red cheek"* from rocky shores of Lake Mweru) were loosely (39% bootstrap support) suggested as the sister clade to *Pseudocrenilabrus*. None of our samples from the upper Congo lakes was related to the haplochromine lineages that gave rise to the radiations in Lakes Victoria and Malawi.

The six species of Lake Bangweulu fall into four divergent haplotype clades: Its population of *Pseudocrenilabrus philander* belongs to a haplotype clade that is widely distributed across southern and eastern Africa, and in fact shares haplotypes with individuals from distant localities. Lake Bangweulu's populations of *Serranochromis robustus* and *S. thumbergi* share the same haplotypes with populations of these species sampled all across southern Africa (Joyce et al. 2005, clade VI). The same is true for the *S. angusticeps* and *S. altus* populations of Bangweulu (Joyce et al. 2005, clade III). The *Sargochromis* of Lake Bangweulu form a recently derived haplotype crown clade within the widespread clade that predominates in this genus (Joyce et al. 2005, clade I). Hence, with the exception of *Sargochromis* sp. "smoky face mellandi", Lake Bangweulu did not reveal any endemic haplotype clades.

Fig. 4: Fig. 4: Maximum likelihood tree (GTR + I + Γ) showing the haplochromine cichlid radiations of Lake Mweru using mitochondrial control region (D-loop). All Lake Mweru haplotypes are highlighted with orange branches, all Bangweulu haplotypes with blue branches, all other haplotypes have black branches. Bootstrap values (only >70%) from 100 pseudoreplicates are shown above relevant branches. For comparison, the radiations of Lakes Victoria, Malawi, Tanganyika, and various East and South African riverine species are shown at the base of the tree. Clades were numbered (I-VI) according to Joyce et al (2005). The *Pseudocrenilabrus* radiation of Lake Mweru was numbered VII, the *Orthochromis* radiation of Lake Mweru was numbered VIII. Four of the new radiations (clades Ia, IIIa, IV, VII) contain individuals that phenotypically belong to a distant clade indicating the possibility of past hybridization. Members of the endemic sub-radiations of Lake Mweru are labelled with colourful squares according to their phenotypic clade affiliations. Lake Mweru individuals that belong to non-radiating groups have no symbols.

Ia Lake Mweru/Bangweulu
Sargochromis

I *Sargochromis*

IIIa Lake Mweru
Serranochromis large tooth

III *Serranochromis altus/angusticeps*

II *Serranochromis* Zambezi

IV Lake Mweru
Serranochromis small tooth

VI *Serranochromis robustus/thumbergi*

V *Thoracochromis buysi/albolabris*

IX Lake Mweru *Orthochromis sp. red fin*
and Lower Congo riverine *O.
polyacanthus* and *T. demeusii*

VII Lake Mweru
Pseudocrenilabrus

Pseudocrenilabrus
Lake Bangweulu

X *Pseudocrenilabrus*
East and South Africa

VIII Lake Mweru *O. kalungwishiensis /
sp. red cheek*

Lake Victoria

Lake Malawi

East African rivers and lakes

Lake Tanganyika haplochromines

Orthochromis Lake Tanganyika drainage

Lamprologini

Astatoreochromis alluaudi (Lake Victoria))
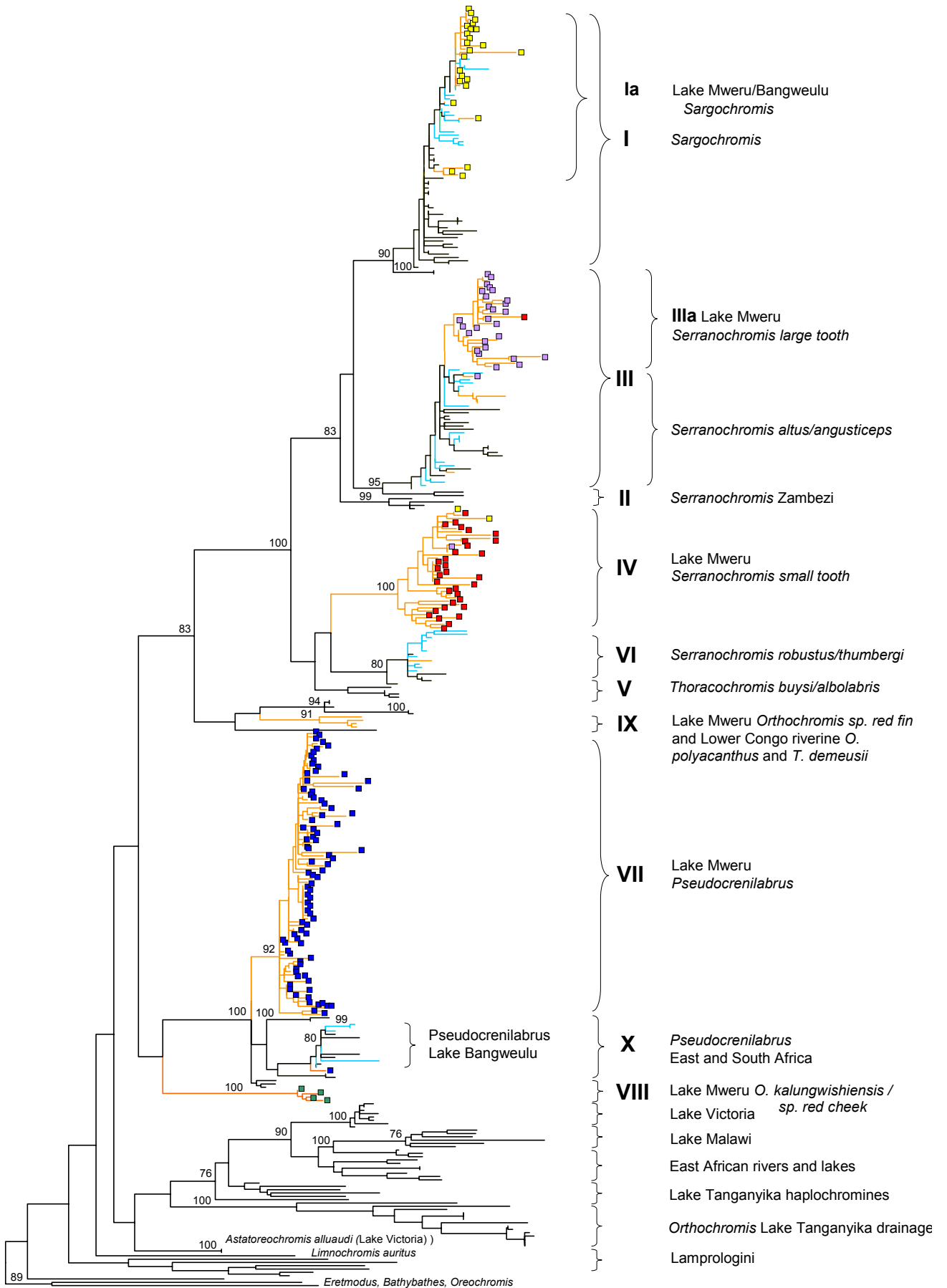*Limnochromis auritus*

Eretmodus, Bathybathes, Oreochromis

Fig. 4

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Our Lake Mweru samples fall into eight distinct haplotype clades. The sequences of the three *Orthochromis* species make two different clades. Both are deeply divergent from all others and from one another. One (*O. sp. "red fin"*, clade IX) is closer to the serranochromines as part of a clade of strictly Congolese haplochromines. The other one (*O. kalungwishiensis* and *O. sp. "red cheek"*, clade VIII) is a new haplotype lineage on its own that is closer to *Pseudocrenilabrus*. It should be noted that neither of these was related at all to the phenotypically similar *Orthochromis* species from the Lake Tanganyika drainage (Salzburger et al. 2005) (Fig. 4). Most of the phenotypically diverse *Pseudocrenilabrus* fall into a distinct, new clade (VII) that contains exclusively Mweru individuals. However, one individual in 86 had a distantly related and unique haplotype that is much more closely related to the Bangweulu/Panafrican clade of *Pseudocrenilabrus* (clade X). This haplotype was found within one of the phenotypically highly derived Mweru species, alongside several haplotypes of the Mweru clade. *Serranochromis robustus* of Lake Mweru carries a haplotype that is part of the *S. robustus/thumbergi* clade from across southern Africa (Joyce et al. 2005, clade VI). Similarly, the populations of *S. angusticeps* from Lake Mweru form one haplotype clade with populations of this species sampled all across southern Africa (Joyce et al. 2005, clade III).

The entire endemic phenotypic diversity of Lake Mweru serranochromines falls into three haplotype clades, each largely reciprocally monophyletic. Two of these are crown clades embedded in haplotype radiations from the Pleistocene palaeo-Lake Makgadikgadi (crown clades in clades I and III; Fig. 4). The third one is a highly distinct clade on its own not known from outside Lake Mweru (clade IV). These three clades correspond largely with the *Sargochromis*, large tooth *Serranochromis* and small tooth *Serranochromis*, respectively, but not completely. Each of the phenotypically defined groups contains a minority of mitochondrial haplotypes of the other clades, suggesting either taxonomic errors, homoplasy of the phenotypes, or hybridization between the very divergent lineages.

Specifically, one *Sargochromis sp. red face* and one *Sargochromis cf. mellandi* (labelled with yellow asterisks in Fig. 5) shared haplotypes with the *Serranochromis* small tooth clade. One *Serranochromis sp. macrocephalus-like* (green asterisk), phenotypically a large tooth *Serranochromis*, had a haplotype of the small tooth *Serranochromis* clade. One *S. sp. checkerboard* (green asterisk), phenotypically also a large tooth *Serranochromis*, had a haplotype of the non-endemic *Serranochromis angusticeps*. One *S. cf. stappersi* (red asterisk), phenotypically a small tooth *Serranochromis*, had a haplotype of the large tooth *Serranochromis*.

Nuclear phylogeny of the Lake Mweru serranochromine sub-radiations and cyto-nuclear discordance analysis

To answer the question if the mismatches between phenotypically defined clade membership and mitochondrial clade membership were due to taxonomic errors (or homoplasy) or due to hybridization, we genotyped the above named five individuals plus eleven other Mweru individuals and two outgroups at 1331 AFLP loci. Reconstructing their phylogeny based on these 1331 mostly nuclear loci (Fig. 5) clearly revealed that hybridization explained the presence of distantly related minority haplotypes in all radiating serranochromine clades of Lake Mweru. All three phenotypically defined clades were highly supported in the AFLP tree, and all five individuals with "foreign" mitochondrial haplotypes clustered well within their respective genomically defined clades. When building a mitochondrial genealogy for the same subset of individuals, the mismatch between the two trees is apparent (Fig. 5). Moreover, in the AFLP tree, the three Lake Mweru serranochromine groups together make a monophyletic radiation.

Hybridization between members of the mitochondrially distantly related clades could have happened recently between already well differentiated species of each sub-radiation. Alternatively, segregation of distantly related haplotypes could be a legacy of hybridization

early during colonization of the lake prior to the extensive speciation. Was hybridization recent, and its signature restricted to the individuals in our data set with haplotype-phenotype mismatch, we would expect these to be resolved in the AFLP tree at the base of those clades whose genome they share most of. The overall relationships between the three radiating clades relative to species that are not part of either of the radiations would not be affected. In contrast, early hybridization between the colonizing lineages before each started to radiate, would leave a genomic signature of reticulate evolution in all members of all three sub-radiations. This might make the three lineages appear more closely related to each other than either is to its ancestors, just like our AFLP tree suggests. The three endemic Lake Mweru sub-radiations (yellow, green and red in Fig. 5) are much more closely related, relative to the relationships of either to other cichlids, in the AFLP tree than in the mitochondrial genealogy. In fact, all endemic radiating clades together appear monophyletic in the AFLP tree, to the exclusion of *Serranochromis angusticeps* and *S. altus* even though these are immediately ancestral to the mitochondrial lineage of the Lake Mweru large tooth *Serranochromis*. Hence, the data suggest ancient hybridization between three deeply divergent colonizing lineages of serranochromines, followed by adaptive radiation within each haplotype clade.

Dating the onset of the four Lake Mweru sub-radiations
Within all four Lake Mweru sub-radiations, branch lengths were short and lineage sorting between species was highly incomplete. The average sequence divergence within sub-radiations was 1.3 % which translates into on average 9 base pair substitutions per sequence (mean pairwise genetic distance between sequences calculated as uncorrected p-distances: clade I: p = 0.012; clade III (only *Serranochromis* large tooth): p = 0.012; clade IV: p = 0.019; clade VII: p = 0.011). Applying two different non-linear molecular clocks to obtain age estimates for the onset of the radiations suggests that three sub-radiations started at quite exactly the same time: In the *Pseudocrenilabrus* clade 180/250 $\pm$ 70/90 ka (mean pairwise estimates of fossil record/Gondwana fragmentation calibration), in the majority-*Sargochromis* haplotype clade 260/370 $\pm$ 130/170 ka, and in the majority-large tooth *Serranochromis* clade 450/700 $\pm$ 90/120 ka. The haplotype radiation in the majority-small tooth *Serranochromis* clade is with 590/940 $\pm$ 120/116 ka slightly older than the others. These estimates suggest that all four haplotype radiations are recent, and that three took place contemporaneously, whereas the radiation in the majority-small tooth *Serranochromis* haplotype began slightly earlier.
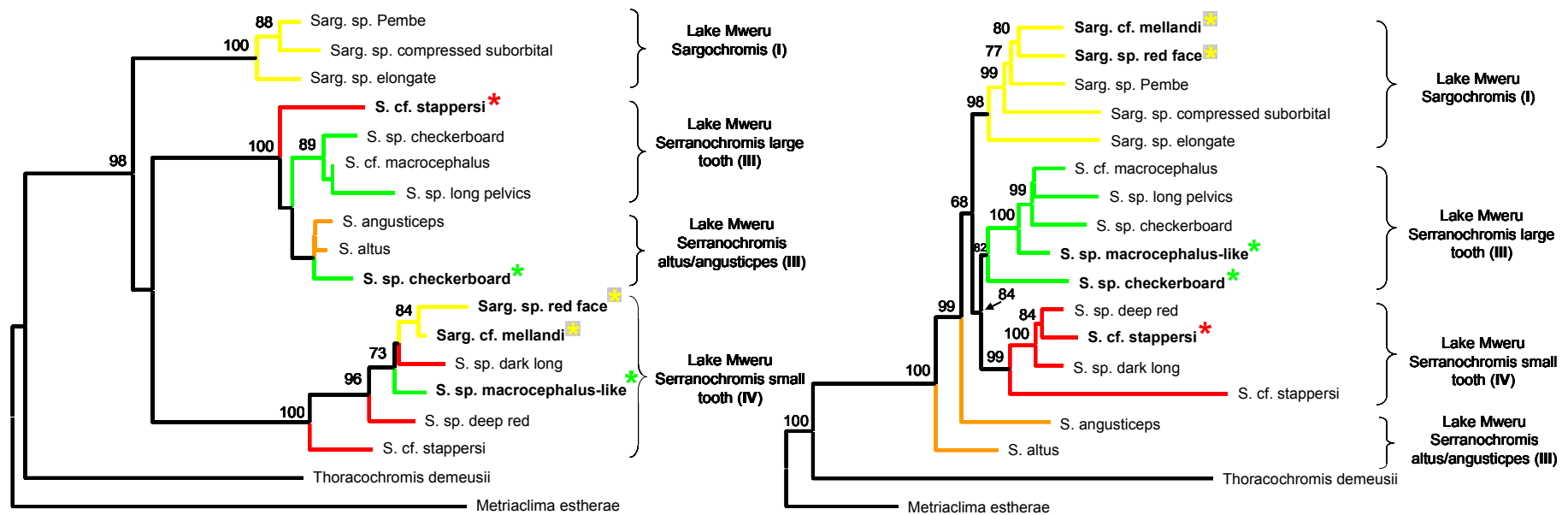
Fig. 5: Cyto-nuclear discordance in five individuals (labelled with asterisks) of the three serranochromine radiations of Lake Mweru. On the left: Neighbour joining tree (GTR + I + Γ) of 706 bp of the mitochondrial control region. On the right: AFLP phylogeny (Fitch-Margoliash) of the same individuals, based on 1331 nuclear loci. Bootstrap values from 1000 pseudoreplicates per tree are shown above branches (only values >70%). Th. demeusii was used as outgroup. On the AFLP tree, all five individuals were assigned to the correct phenotype clade despite sharing mtDNA haplotypes with a distantly related, phenotypically dissimilar clade. Clade numbers correspond to Fig. 4

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Dating the divergence between the colonizing lineages of Lakes Mweru and Bangweulu
The divergence between the four to six haplochromine lineages that colonized Lake Bangweulu ranged from 2.43/3.07/5.89 MY to 4.70/9.71/21.26 MY (mean pairwise estimates of the internal/ fossil record/Gondwana fragmentation calibration) uncorrected p-distances = 0.049 to 0.095; all results from pairwise radiation comparisons in Supplementary Table 1).

  The divergence between the eight lineages that colonized Lake Mweru ranged from 1.70/1.65/2.94 MY to 4.95/10.60/23.46 MY, uncorrected p-distances = 0.034 to 0.100). Our relaxed molecular clocks suggest 3.11/4.72/9.51 MY divergence between small tooth *Serranochromis* and large tooth *Serranochromis* (uncorrected p-distance = 0.063), 2.90/4.20/8.34 MY divergence between small tooth *Serranochromis* and *Sargochromis* (uncorrected p-distance = 0.059)*,* and 2.52/3.27/6.32 MY of divergence between large tooth *Serranochromis* and *Sargochromis* (uncorrected p-distance = 0.051). The two ancient *Pseudocrenilabrus* lineages that hybridized in Lake Mweru are somewhat less divergent, but they are still separated by 1.70/1.65/2.94 MY divergence time (uncorrected p-distance = 0.034).

Morphological analysis: species differentiation in Lake Mweru
"Species" as a factor explained highly significant parts of the variance in the first three PC axes of morphology in each of the four sub-radiations (Supplementary Table 2). The *Sargochromis* sub-radiation (clade I) was composed of medium-sized (adult size range between 10 and 25 cm), rather deep bodied fishes with trophic adaptations ranging from insectivorous to specialized snail crusher morphology (Plate 1 in Supplementary Material). All species were found in littoral habitats, mostly near submerged or emergent vegetation in lagoons and along the shores of the main lake. We had visually assigned individuals to eleven different species in the field (Supplementary Table 2, Fig. 6 a,b). Most of these were confirmed as distinctly different using linear morphometric distances. 62%, 43% and 29% of all posthoc tests comparing species in PC1, PC2, and PC3, respectively, were significant. *Sarg.* sp."big miller", *Sarg.* sp. Chief's Pond*, Sarg.* sp. Pembe, *Sarg.* sp."yellow face small teeth" were significantly different from all other species. *Sarg. cf. mellandi*, *Sarg.* sp. "yellow face big teeth", and *Sarg.* sp. "red face" showed considerable overlap along all axes. Whereas *Sarg.* sp. "red face" appears distinct nevertheless in its colour, it is possible that *Sarg. cf. mellandi* and *Sarg.* sp. "yellow face big teeth" are conspecific. Among the rare species of which we obtained single specimens, three are phenotypically very distinct. Hence, we conclude that we collected at least 9 putative species in this sub-radiation.

  The small tooth *Serranochromis* sub-radiation (clade IV) was composed of medium sized to rather large predators with diverse morphologies and ecology (Plate 1 in Supplementary Material). Some are littoral ambush hunters, others are offshore pelagic predators, and one resembles morphologically the peadophages of Lake Malawi and Lake Victoria. 30%, 50%, and 30% of all posthoc tests comparing species in PC1, PC2, and PC3, respectively, were significant. *S.* cf. stappersi, *S.* sp. "silver long", and *S.* sp. "dark long" were significantly different from all other putative species (Supplementary Table 2, Fig, 6 c,d). *S.* sp. "deep red" and *S.* sp. "diplotaxodon face" were not different from one another in the morphometrics, but had very different colour and habitat. Hence, we conclude that we documented at least six putative species.

  The large tooth *Serranochromis* sub-radiation (clade III) was composed of medium sized to very large (> 25cm total length) fish predators (Plate 1 in Supplementary Material). They were found both inshore near submerged and emergent vegetation, and offshore in shallow areas over soft bottom. The four species of which we had population samples, formed distinct, barely overlapping clusters in morphospace (Supplementary Table 2, Fig. 6 e,f). 100%, 100%, and 50% of all posthoc tests comparing species in PC1, PC2, and PC3, respectively, were significant. Some of the species differed additionally in their PC3 scores. *S.*

sp. "long face blue", of which we had only one specimen, overlapped with *S.* sp. "long face yellow", but is distinct in male nuptial colour. Hence, we conclude that we collected 5 putative species in this sub-radiation.

The *Pseudocrenilabrus* sub-radiation (clade VII) was composed of very small (3 cm total length) to small species, none exceeding 10cm in total length (Plate 1 in Supplementary Material). They occupied all sampled habitats from littoral vegetation to rocky boulder shores and the offshore demersal and pelagic zones. We had visually assigned individuals to 19 different species in the field, including the ancestral widely distributed *P. philander* phenotype. The quantitative morphological analysis supported the distinctiveness of most of these (Supplementary Table 1, Fig. 7). 47%, 10%, and 46% of all pairwise comparisons between species were significantly different along PC1, PC2, and PC3, respectively. To further investigate the different habitat groups within the *Pseudocrenilabrus* sub-radiation and for better visual resolution, we calculated additional ANOVAs on species from each major habitat separately: Within the sand and weed dwellers (7 putative species, Fig. 7 a,b) and within the offshore demersal/pelagic group (7 putative species, Fig. 7 c,d) species explained significant fractions of the variance and many species were significantly different from one another. In the demersal/pelagic group in particular, male nuptial coloration was bright and diversified. Within the rocky shore dwellers (nominally members of the genus *Thoracochromis*), no significant differences in morphology were detected between the four putative species (Fig. 7 e,f). However, all four differed conspicuously in male nuptial dress (Plate 1 in Supplementary Material). We conclude that we had indeed collected at least 15 putative *Pseudocrenilabrus* species.

Morphological analysis: radiation-wide patterns
Comparison of the Lake Mweru radiation with the three classical East and South African adaptive radiations of haplochromine cichlids reveals that the Lake Mweru radiation occupies a large area in morphospace, directly comparable in volume to the other radiations (Fig. 8). ANOVA with 'radiation' as factor (Mweru, Victoria, Malawi, Makgadikgadi) and subsequent Tukey-Kramer tests showed that some of the radiations had their centre in significantly different in the positions in morphospace (Supplementary Table 2; see also Young et al.; Joyce et al. 2005). Along PC1, the radiation of Lake Victoria differed from all others. Along PC2, the Lake Malawi radiation differed from all others. The Lake Mweru radiation did not differ from that of palaeo-Lake Makgadikgadi.

Comparing the four Lake Mweru sub-radiations reveals almost perfectly complementary distributions in morphospace (Fig. 9). The first two major axes of morphological variance, PC1 and PC2, both revealed 83% significant differences between sub-radiations (Supplementary Table 2). In posthoc pairwise radiation comparisons, the *Pseudocrenilabrus* species flock showed significant differences from all other Lake Mweru sub-radiations along PC1 and 2. The *Sargochromis* sub-radiation was different from all other sub-radiations along PC axis 1, whereas the small tooth *Serranochromis* sub-radiation differed from all other sub-radiations along PC 2.
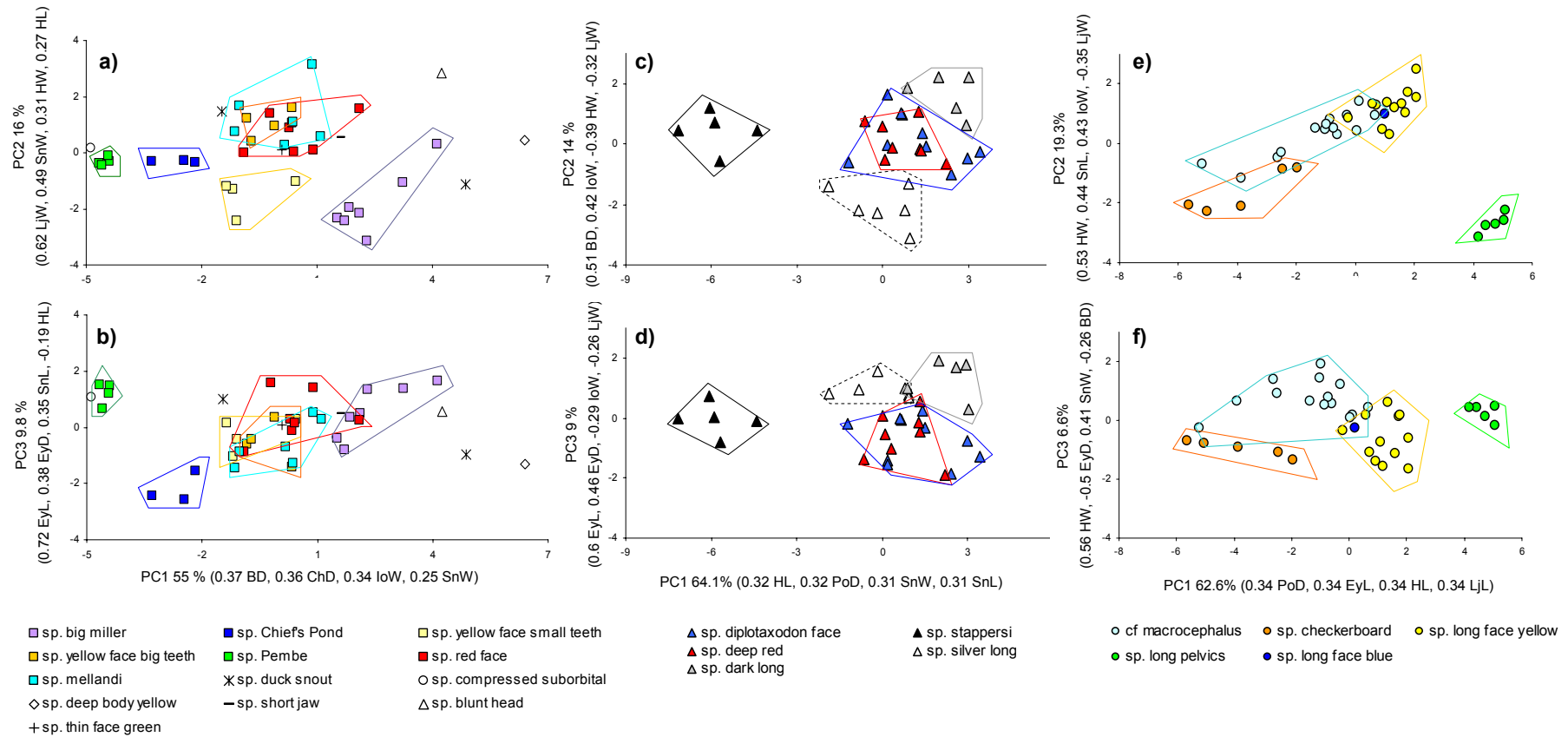
Fig. 6 a-b): Morphological analysis of the *Sargochromis* sub-radiation (clade I) of Lake Mweru using principal component analysis (PCA) on 13 standardized log-transformed and size-adjusted morphological traits. Upper gtaphs show PC2 scores on PC1 scores, lower graphs show PC3 scores on PC1 scores. Coloured polygons show the areas in morphospace occupied by the putative species. Every data point represents an individual. Axes show principal components 1, 2 (top) and 3 (bottom) with percentage of variance explained by each component. Brackets contain the eigenvalues of the four traits with the highest loadings. c-d) Analysis of the small tooth *Serranochromis* sub-radiation (clade IV) of Lake Mweru. e-f) Analysis of the large tooth *Serranochromis* sub-radiation (clade III) of Lake Mweru.
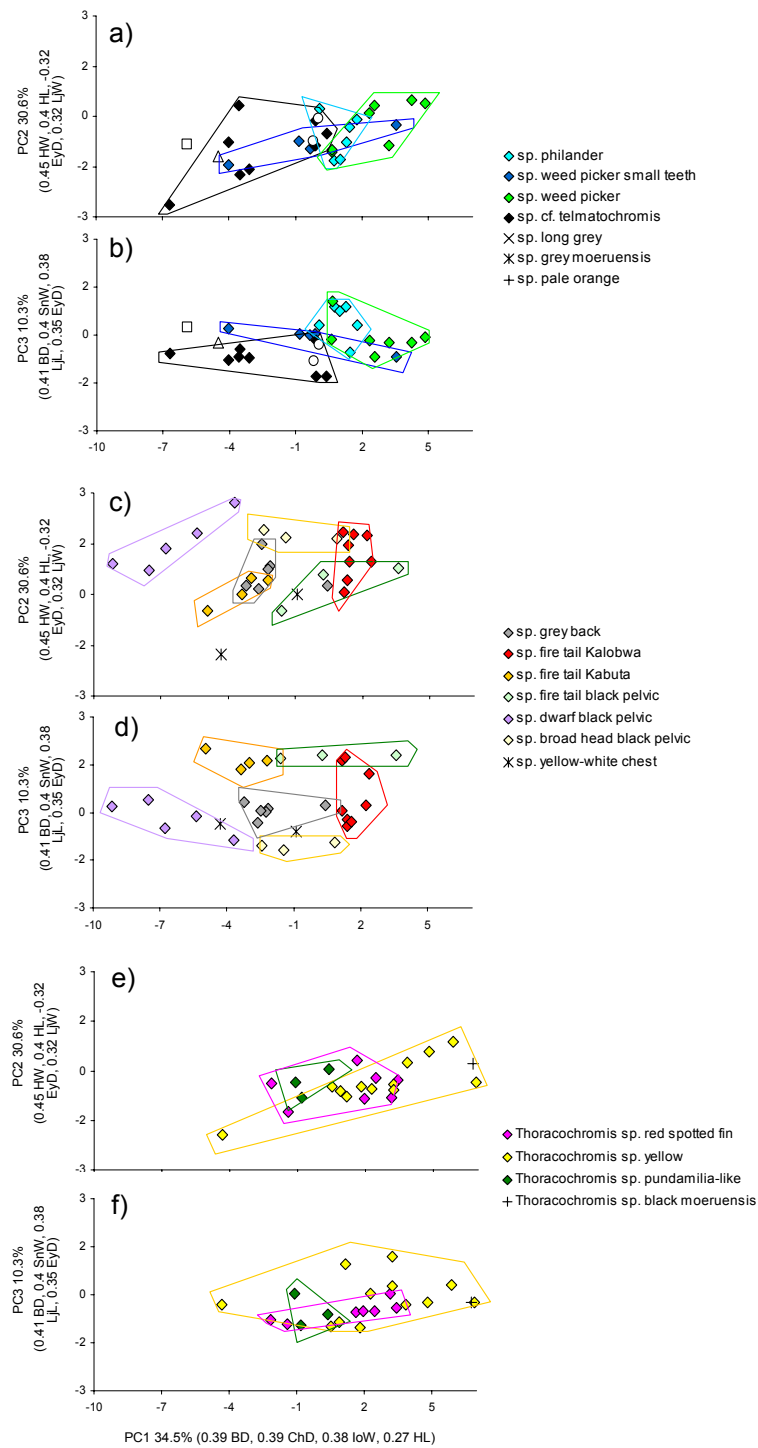
Fig. 7: Morphological analysis of the *Pseudocrenilabrus* radiation of Lake Mweru using principal component analysis (PCA) on 13 standardized log-transformed and size-adjusted morphological traits. Coloured polygons show the areas in morphospace occupied by the putative species. Every data point represents an individual. Axes show principal components 1, 2 (top) and 3 (bottom) with percentage of variance explained by each component. Brackets contain the eigenvalues of the four traits with the highest loadings. For better visual resolution of each species, the total morphospace of the *Pseudocrenilabrus* radiation was broken up into the three trophic groups. a)- b) the littoral, sand- and weed-dwelling ecotypes, c)-d) the offshore pelagic and demersal feeders, e)-f) the rocky shore dwellers.
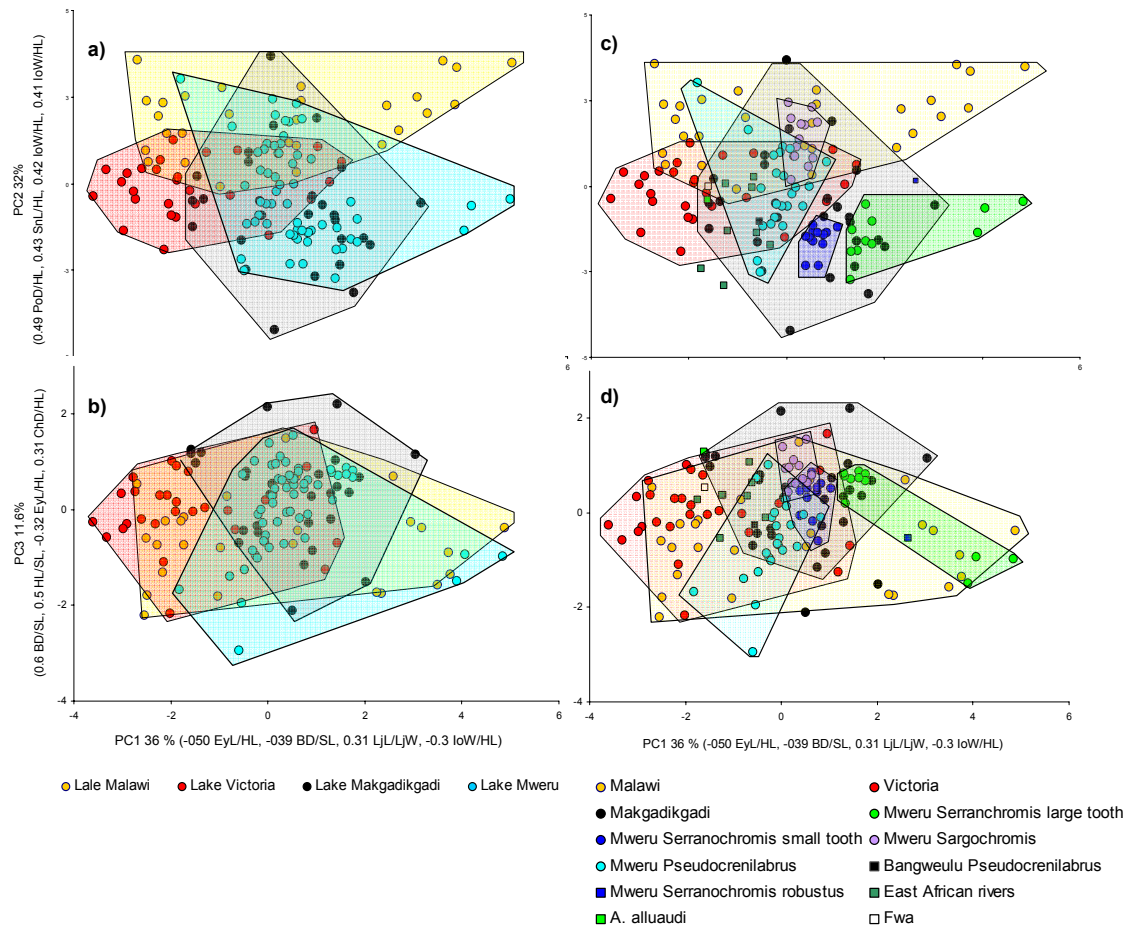
Fig. 8 a)-b) Analysis of the total morphospace occupied by the radiations of Lake Mweru compared to those of Lake Victoria, Malawi and palaeo-Makgadikgadi using principal component analysis. Coloured polygons show the areas in morphospace occupied by each radiation. Every data point represents a different species/population. Data was averaged over at least three individuals per group. c)-d) Same, but the morphospace of Lake Mweru is broken up into the four intralacustrine radiations. Also shown are various species from North and East African lakes and rivers and additional Lake Mweru species not part of the radiations.

DISCUSSION

Adaptive radiation of a single species into many ecologically different species is an important process in the evolution of species and ecological diversity (Schluter 2000). One goal of adaptive radiation research is to identify the roles of extrinsic environmental and intrinsic organismal control of rates of diversification. Here we demonstrate a case in which multiple related colonizing lineages undergo adaptive radiation simultaneously in the same new environment, co-evolving through genetic exchange and ecological interaction.

The diversity of cichlid fish in African lakes and rivers is one of the best model systems for research into these questions (Kocher 2004; Seehausen 2006). Lakes Mweru and Bangweulu are two of the 10 African Great lakes that together straddle a region of major tectonic activity with shifting boundaries between Africa's three major watersheds Nile, Congo and Zambezi (Supplementary Fig. 1). Haplochromine cichlids are widely distributed, but not diverse, in the rivers of the region. Four of the lakes (Malawi, Victoria, Tanganyika, Edward) host large adaptive radiations of haplochromines that are the largest known animal radiations at all. Three others have small radiations (Kivu, Albert, Turkana). Mweru and Bangweulu were thought to have no radiations. The situation is unclear for Rukwa.
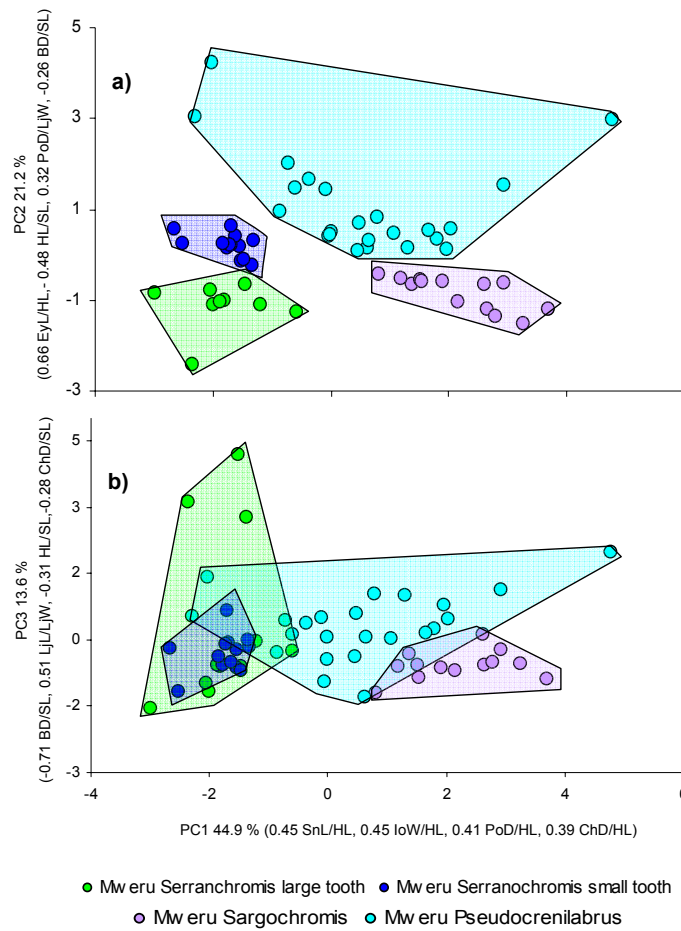
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Fig. 9: Same as Fig. 10, but showing the results of morphospace analysis of only the four Lake Mweru sub-radiations.

We investigated Lakes Mweru and Bangweulu. Multiple non-endemic riverine haplochromine species were known to populate both lakes. At least Lake Mweru is relatively well isolated and diverse in habitats, comparable to those great lakes that have large radiations. It is 6th in size among the great lakes, with 4400 km^2 larger than Lake Edward (2600km^2) which has a radiation of 60 species. Area-wise Lake Mweru and Bangweulu both lie on the steeply ascending arm of the evolutionary species-area relationship for African cichlids (Seehausen 2006). The geology of Lake Mweru is not well studied, but it appears the lake is of early Pleistocene origin. Tectonic activity which led to capture of several upper tributaries of the Zambezi River by the Congo River 1.2-1.8ma BP (Moore and Larkin 2001) are thought to have created a larger precursor of Lake Mweru, which alongside Bangweulu, reached its maximum in the Pleistocene (Dixey 1944). Hence, the conditions seemed favorable for adaptive radiation of cichlids. One difference between Lakes Mweru and Bangweulu is in their drainage history. The rivers feeding modern Lake Mweru have a mixed water shed history. Some have always been Congo (Luapula) drainage, but the Luapula itself captured the upper Kafue-Zambezi 1.2-1.8ma, and hence, Lake Mweru became accessible to Zambezi fauna in the Pleistocene. The Bangweulu drainage on the other hand, has been Zambezian, until the capture event, subsequent to which it probably remained rather isolated from the downstream Luapula/Mweru/Congo system by a series of falls and major rapids.

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We collected cichlids from a large number of sites and all available habitats in both Lake Mweru and Bangweulu. We investigated the phenotypic and ecological diversity, phylogenetic relationships, temporal dynamics of colonization and diversification and performed experiments on behavioural reproductive isolation of species in one of the radiations. We discovered a major adaptive radiation in Lake Mweru, comparable in size to that of Lake Edward. On the other hand, we could not find evidence for any radiation in Bangweulu. The haplochromine fauna of Lake Mweru is a composite of Congolese (clades VIII, IX), endemic (clades IV, VII) and Zambezian (clades I, III, VI) mitochondrial lineages. That of Bangweulu on the other hand is exclusively Zambezian (clades I, III, VI, X). Yet, the species diversity in Lake Mweru is not ancient, but very recent. In fact, the haplotypic radiations endemic to Lake Mweru are mostly younger than the haplotypic radiations in Lake Bangweulu. Yet, they are associated with species radiations in the former, but not in the latter.

For Lake Bangweulu, we found no evidence for hybridization between colonizing lineages, except perhaps between the two most closely related species, *Serranochromis altus* and *S. angusticeps*, which in fact may have originated and speciated in this lake, in which case they could be considered a minor radiation in Bangweulu (2-3 species, Plate 1 in Supplementary Material). In Lake Mweru on the other hand, we find strong evidence for ancient hybridization between several distantly related colonizing lineages. Experimental work on the rates of accumulation of genetic incompatibilities among African haplochromine cichlids suggests that compatibility is becoming very low after 2/4/8 MY (depending on the clock used: internally calibrated linear/fossil plus recent geology nonlinear/Gondwana plus recent geology nonlinear) of divergent evolution and is completely lost after 4/8/15 MY (Stelkens et al. submitted). Hence, many of the lineages that colonized Mweru are that divergent that they fall outside this window of opportunity (19 lineage pairs with divergence >3.5/5/10 MY in Table 1). Indeed we did not find any evidence for hybridization between these. Nine other pairs, involving seven lineages, fall within this broadly defined window of opportunity. We find that ancient hybridization has occurred between four of these, involving five different lineages. All of these are associated with adaptive species radiations, whereas we discovered no sign of species radiations in any of the lineages that were not implicated in hybridization. The only pairs that may not be fully postzygotically isolated but for which no evidence for hybridization was found, were the *Orthochromis kalungwishiensis* clade versus *Pseudocrenilabrus*, and the serranochromine pairs including the very rare *S. robustus*.

The interaction between hybridization and adaptive radiation lead to two genetically fully independent sub-radiations, one involving 1.7/1.7/2.9 MY divergent lineages of *Pseudocrenilabrus*, the other one involving three different 2.5-.3/3.3-4.7/6.3-9.5 MY divergent lineages of serranochromine cichlids. Hybridization must have been ancient, probably before the species radiations started, as implicated by nuclear genomic monophyly of the Mweru serranochromine radiation despite very deep polyphyly in the constituent mitochondrial lineages. In the case of *Pseudocrenilabrus*, recent introgression can be excluded because the divergent mitochondrial lineage that is rare in Lake Mweru *Pseudocrenilabrus* is unique, but shares an ancient sister relationship with the haplotype clade now widespread in the Zambezi system and Lake Bangweulu. The large difference in the frequencies of the two haplotype clades within Mweru *Pseudocrenilabrus* suggest that the majority haplotype clade is on its way to fixation. Similar patterns have been documented in other fish species pairs of radiations following hybridization of distinct mitochondrial lineages (e.g. in cyprinids (DeMarais *et al.* 1992) and sticklebacks (Taylor and McPhail 2000)).

Lake Mweru's large diversity in ecologically relevant shape variation resembles that of the classical cichlid adaptive radiations. Quantitative morphological analysis suggests that each of the four new sub-radiations contains between five and 15 phenotypically distinct taxa, many exhibiting novel phenotypes not reported from anywhere else in the large distribution

range of the founding lineages. The latter is particularly true for *Pseudocrenilabrus* and "small tooth *Serranochromis*". At the same time, some of the Lake Mweru species closely resemble taxa of unrelated lineages in other African radiations. *Pseudocrenilabrus* sp. "pundamilia-like" for example, resembles in astonishing detail the Lake Victoria cichlid genus *Pundamilia* (Seehausen 1996). This is consistent with the parallel evolution of convergent sets of 'ecomorphs' in similar environments, which has previously been demonstrated for other cichlid radiations (Fryer and Iles 1972; Greenwood 1975; Kocher et al. 1993; Seehausen et al. 2003) and many other organisms (Schluter 1996; Losos et al. 1998; Bossuyt and Milinkovitch 2000; Madsen et al. 2001; Gillespie 2004). Notwithstanding their large morphological disparity all species within all sub-radiations are closely related and lineage sorting is absent, consistent with a recent, selection-driven increase in the morphological and ecological diversity of a rapidly multiplying lineage (Schluter 2000).

Age calibration of the onset of each radiation, using two independent cichlid-specific relaxed molecular clocks and an internally calibrated clock, suggests at least three of the sub-radiations occurred largely simultaneously between $180/250 \pm 70/90$ k and $450/700 \pm 90/120$ k BP. The sub-radiation of small tooth is slightly older $590/940 \pm 120/116$ ka. This is the first case of multiple adaptive radiations of cichlids occurring roughly at the same time in the same lake under fully sympatric conditions. Except for a small radiation of tilapiines (3-4 spp) besides the large haplochromine radiation (> 500 spp) in Lake Malawi, all other, comparably recent cichlid radiations (Lakes Victoria, Edward, Malawi), albeit probably being a genetic admixture of several colonizing species, represent but one radiation (Sturmbauer et al. 2001; Seehausen et al. 2003; Won et al. 2006). The only other case of multiple lineages radiating in the same lake is the polyphyletic cichlid assemblage of Lake Tanganyika, but it seems the main radiations have taken place consecutively rather than simultaneously there (Takahashi et al. 2001; Salzburger et al. 2002; Salzburger et al. 2005). Whereas the volume in morphospace in the Lake Mweru radiation overall resembles that in the classical cichlid radiations (Fig. 8), it is internally strongly structured by colonizer lineage. The two main radiations, and also the three sub-radiations within the serranochromine radiation, complement each other in morphospace with astonishing precision and practically no overlap between them (Fig. 9). This is likely the result of divergent ecological selection driving phenotypic evolution along different axes of shape variation in each radiation, generating different feeding types. *Serranochromis* contains exclusively predatory species, large piscivores and ambush hunters with relatively long heads and long, narrow jaws. *Sargochromis* is represented mainly by insectivores and molluscivores with deep heads and enlarged pharyngeal jaw bones required for snail crushing. *Pseudocrenilabrus* contains only small-bodied species that are planktivores, algae scrapers or suckers, with a diverse array of specialized jaw and tooth morphologies. Such eco-morphological complementation could arise either through competitive exclusion, through different developmental or constructional constraints, or through an interaction of both. Developmental or constructional constraints alone seem unlikely to generate the precise complementation between lineages that we demonstrate (Fig. 9). Evidence for competitive exclusion also comes from comparison of the serranochromine phenotype diversity with that in the serranochromine radiation of palaeo- Lake Makgadikgadi (Joyce et al. 2005). The seeding lineages of both lake radiations are very closely related. Yet, Lake Mweru serranochromines lack a large section of the palaeo-Lake Makgadikgadi morphospace volume, which in Mweru is filled instead by *Pseudocrenilabrus* species. Hence, we conclude that cichlid adaptive radiation is indeed constraint by the presence of competing lineages.

Joyce et al. (2005) observed that some trophic types, common in the classical Great Lakes radiations, were missing from the Makgadikgadi radiation. These were specialists of the pelagic open waters, including pelagic predators and zooplanktivores, requiring shallow bodies, broad heads and large eyes, and also species with short wide heads and jaws that

scrape algae from rocks (epilithos-feeders). They speculated that this was due to a lack of ecological opportunity in the mostly riverine habitat that remnants of the palaeo-Lake Makgadikgadi radiation now occupy. If this hypothesis was correct, we expected to find such trophic types in the Lake Mweru radiation. All of the 'missing' trophic types do indeed exist in Lake Mweru, but only some, namely pelagic predators evolved within the serranochromines. The other two trophic types, involving small particle feeding, evolved in multiple versions within the *Pseudocrenilabrus* radiation but not in the serranochromines. Given that serranochromines are predominantly predators wherever they occur in Africa, whereas *Pseudocrenilabrus* are small particle feeders everywhere, the direction of evolutionary complementation observed in Lake Mweru, and the direction of character displacement between Lake Makgadikgadi and Mweru, are probably the product of an interaction between historical constraint and ecological interaction.

The Lake Mweru radiation is unique among African cichlid radiations in several ways. First, the genus *Pseudocrenilabrus*, impressively diversified in Lake Mweru, also occurs in all other African Great lakes, but has not speciated anywhere else and is a typically seen as a non-radiating haplochromine (Seehausen 2006). Second, next to the amazing radiation in *Pseudocrenilabrus*, Lake Mweru also accommodated a large radiation of another totally different lineage. Other lakes typically have only one radiating lineage. One reason for this distinction could be that the starting conditions in Lake Mweru were different from those in all the other lakes. While the distantly related founders of the Lake Victoria and Malawi radiations were ecologically and morphologically similar species of one major lineage (*Astatotilapia*; Joyce et al. 2005), the multiple colonists of Lake Mweru started out as phenotypically and ecologically divergent types. It seems plausible that the initial occupation of different niches in the newly formed lake allowed for coexistence of most ancestral types, as still seen today in Lake Bangweulu, providing then several different starting points in niche space for subsequent episodes of adaptive radiation. Competition for resources between the main ecological guilds may have then constrained the directions and set the boundaries to phenotypic diversification within each radiation.

However, the same should in principle have been possible in Lakes Victoria, Malawi, Edward and Tanganyika. Each of these lakes was colonized by several very deeply divergent haplochromine lineages besides the one that radiated: *Pseudocrenilabrus* colonized all lakes, *Serranochromis* colonized Lakes Malawi and Tanganyika, *Astatoreochromis* colonized Lakes Victoria, Edward and Tanganyika. Yet none of these colonists radiated. One difference is that these lakes, in contrast to Lake Mweru, were colonized by just one species of each lineage, except for multiple *Astatotilapia/"Thoracochromis"*. If hybridization between distant relatives was to jump-start haplochromine radiations, then the only lineage that had this opportunity in all the other lakes was indeed *Astatotilapia* (including the nilotic *"Thoracochromis"*). Divergence time between all the other colonists (Genner et al. 2007) falls well outside the experimentally determined window of opportunity for hybridization (Stelkens et al. submitted). The same argument would potentially explain why *Pseudocrenilabrus* radiated in Lake Mweru but not in Lake Bangweulu.

Together our data suggest that the radiations of Lake Mweru are recent and that speciation is likely a result of adaptation to the highly heterogeneous lake habitat. Yet, similar opportunity is not associated with radiation in neighbouring Lake Bangweulu. Every radiating Mweru clade experienced ancient hybridization with genetically distant lineages, while no trace of such was found in Lake Bangweulu cichlids. We hypothesize that the resulting increase of genetic variation facilitated diversification and speciation, while ecological interactions between colonising lineages set boundaries to the phenotypic diversity of each.

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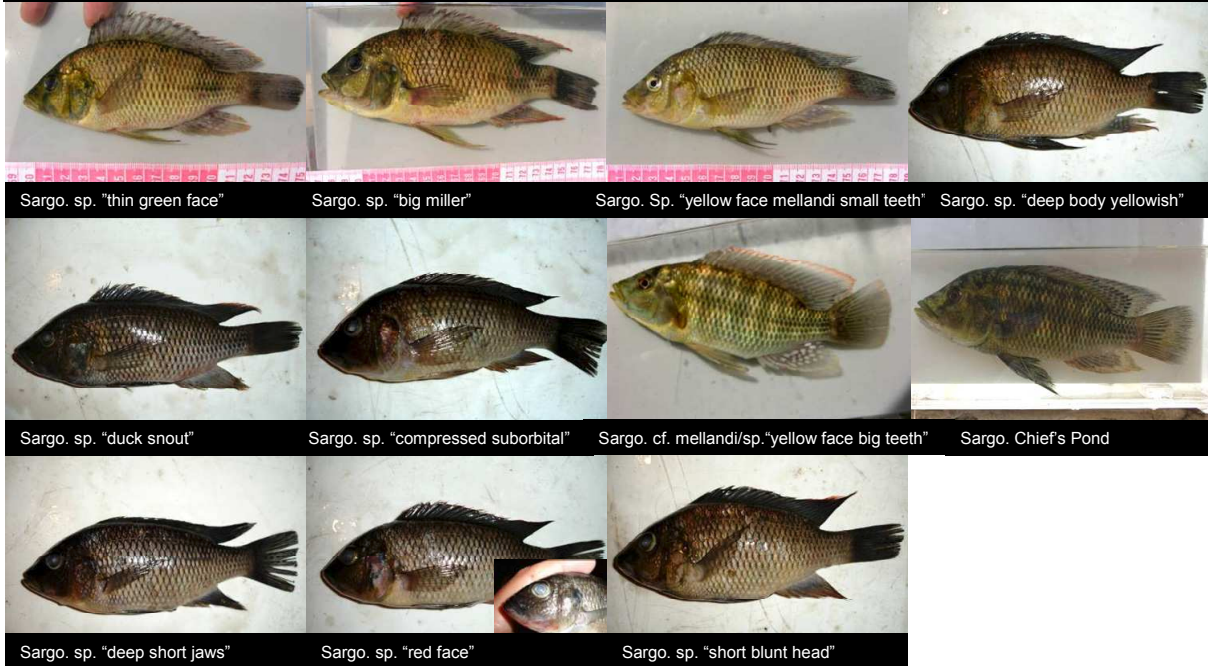
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Sargochromis and Serranochromis Lake Mweru

SARGOCHROMIS

Sargo. sp. "thin green face"
Sargo. sp. "big miller"
Sargo. Sp. "yellow face mellandi small teeth"
Sargo. sp. "deep body yellowish"

Sargo. sp. "duck snout"
Sargo. sp. "compressed suborbital"
Sargo. cf. mellandi/sp."yellow face big teeth"
Sargo. Chief's Pond

Sargo. sp. "deep short jaws"
Sargo. sp. "red face"
Sargo. sp. "short blunt head"

SERRANOCHROMIS LARGE TOOTH

Serrano. sp. "long face blue"
Serrano. sp. "long yellow face"
Serrano. sp. "long pelvics"
Serrano. sp. "checkerboard"

Serrano. cf. macrocephalus 1
Serrano. sp. "Chief's Pond"
Serrano. angusticeps

SERRANOCHROMIS SMALL TOOTH

Serrano. sp. "dark long body"
Serrano. sp. "almost paedophage"
Serrano. sp. "diplotaxodon face"

Serrano. sp. "silver long body"
Serrano. cf. stappersi
Serrano. sp. "deep red"

Pseudocrenilabrus Lake Mweru

SANDY / WEEDY LITTORAL

P. sp. "philander ancestral"

P. sp. "pale orange"

T. sp. "grey moeruensis"

P. sp. "long grey"

P. sp. "weed picker small teeth"

P. sp. "weed picker" (yellow morph)

P. sp. "weed picker" (green morph)

P. sp. " telmatochromis-like pop.2"

P. sp. "telmatochromis-like pop.1"

PELAGIC / DEMERSAL

P. sp. "fire tail Kalobwa"

P. sp. "fire tail black pelvic"

P. sp. "broad head black pelvic"

P. sp. "dwarf black pelvic"

P. sp. "fire tail Kabuta"

P. sp. "grey back"

ROCKY SHORES (nominal genus Thoracochromis)

T. sp. "red spotted fin"

T. sp. "moeruensis black"

T. sp. "pundamilia-like"

T. sp. "moeruensis yellow"

Orthochromis Lake Mweru

O. kalungwishiensis

O. sp. "red cheek"

O. sp. „ red fin"

Lake Bangweulu

Serrano. altus

Serrano. altus x angusticeps ?

Serrano. angusticeps

Serrano. thumbergi

Serrano robustus

Sargo. sp. „smokey face mellandi"

Pseudocrenilabrus philander

Supplementary Fig. 1: Map from Fryer and Iles (1977), showing the shifting boundaries between Africa's three major watersheds Nile, Congo and Zambezi. Indicated are some of the major tectonic events in the region of the African Great Lakes, including Lakes Mweru and Bangweulu. Numbers attempt to illustrate the events in the order in which they took place in a given area (although not strictly chronologically).

Supplementary Table 1: a) uncorrected p-distances (mean pairwise distances of all individuals) and b) corresponding divergence times (in millions of years) between the eight lineages that colonized Lake Mweru using internal (int), fossil record (foss), and Gondwana fragmentation (Gond) calibration. Lineages that hybridized are bold.

a)

	Orthochromis kalungwishiensis/ O. sp.red cheek (Clade VIII)	Orthochromis polyacanthus/ O. sp.red fin (Clade IX)	Pseudocrenilabrus Bangweulu (Clade X)	Pseudocrenilabrus Mweru (Clade VII)	Serranochromis robustus / S. thumbergi (Clade VI)	Serranochromis small tooth (Clade IV)	Serranochromis large tooth / S. altus / S. angusticeps (Clade III)
Orthochromis polyacanthus/ O. sp.red fin (Clade VIII)	0.08276	-	-	-	-	-	-
Pseudocrenilabrus Bangweulu (CladeXx)	0.060877	0.087815	-	-	-	-	-
Pseudocrenilabrus Mweru (Clade VII)	0.05992	0.083916	**0.034301**	-	-	-	-
Serranochromis robustus / S. thumbergi (Clade VI)	0.092025	0.080484	0.088764	0.088143	-	-	-
Serranochromis small tooth (Clade IV)	0.099794	0.08465	0.100058	0.093939	0.053883	-	-
Serranochromis large tooth / S. altus / S. angusticeps (Clade III)	0.089668	0.085794	0.095097	0.091512	0.064321	**0.062833**	-
Sargochromis (Clade I)	0.090732	0.080756	0.093264	0.08805	0.049056	**0.058724**	**0.050903**

b)

	Orthochromis kalungwishiensis/ O. sp.red cheek (Clade VIII)			Orthochromis polyacanthus/ O. sp.red fin (Clade IX)			Pseudocrenilabrus Bangweulu (Clade X)			Pseudocrenilabrus Mweru (Clade VII)			Serranochromis robustus / S. thumbergi (Clade VI)			Serranochromis small tooth (Clade IV)			Serranochromis large tooth / S. altus / S. angusticeps (Clade III)		
	int	foss	Gond	int	foss	Gond	int	foss	Gond	int	foss	Gond	int	foss	Gond	int	foss	Gond	int	foss	Gond
Orthochromis polyacanthus/ O. sp.red fin (Clade VIII)	4.09	7.62	16.23																		
Pseudocrenilabrus Bangweulu (CladeXx)	3.01	4.47	8.95	4.34	8.45	18.21															
Pseudocrenilabrus Mweru (Clade VII)	2.96	4.35	8.68	4.15	7.81	16.68	**1.70**	**1.65**	**2.94**												
Serranochromis robustus / S. thumbergi (Clade VI)	4.55	9.17	19.94	3.98	7.26	15.38	4.39	8.61	18.60	4.36	8.51	18.34									
Serranochromis small tooth (Clade IV)	4.94	10.56	23.34	4.19	7.93	16.96	4.95	10.60	23.46	4.65	9.50	20.76	2.67	3.61	7.06						
Serranochromis large tooth / S. altus / S. angusticeps (Clade III)	4.44	8.76	18.97	4.24	8.11	17.41	4.70	9.71	21.26	4.53	9.08	19.73	3.18	4.92	9.96	**3.11**	**4.72**	**9.51**			
Sargochromis (Clade I)	4.49	8.94	19.40	3.99	7.30	15.48	4.61	9.38	20.47	4.36	8.49	18.31	2.43	3.07	5.89	**2.90**	**4.20**	**8.34**	**2.52**	**3.27**	**6.32**

Supplementary Table 2: Results of one-way ANOVAs testing the morphological disparity between species within the four radiations of Lake Mweru. The last two rows show the results of radiation comparisons within Lake Mweru and comparing Lake Mweru with other East and South African radiations. Principal components 1, 2 and 3 were used as dependent variables, and 'species' or 'radiation' as factor. The three columns on the right show the results of Tukey-Kramer posthoc tests analyzing between species (or radiation) differences.

radiation	principal component	df	F	p	n of species/ radiations	n of possible pairwise comparisons	n of significant pairwise comparisons	total % of significant pairwise comparisons
Lake Mweru *Sargochromis* (clade I)	PC1	6, 34	36.21	< 0.001	7	21	13	62
	PC2		14.36	< 0.001			9	43
	PC3		6.72	< 0.001			6	29
Lake Mweru *Serranochromis* large tooth complex (clade III)	PC1	3, 36	45.88	< 0.001	4	6	6	100
	PC2		61.13	< 0.001			6	100
	PC3		12.96	< 0.001			3	50
Lake Mweru *Serranochromis small tooth complex* (clade IV)	PC1	4, 33	41.28	< 0.001	5	10	3	30
	PC2		19.86	< 0.001			5	50
	PC3		12.38	< 0.001			3	30
Lake Mweru *Pseudocrenilabrus* (clade VII)	PC1	12, 76	50.53	< 0.001	13	78	37	47
	PC2		4.89	< 0.001			8	10
	PC3		19.22	< 0.001			36	46
Lake Mweru *Pseudocrenilabrus* sand- & weed- dwellers	PC1	3, 25	3.88	0.023	4	6	1	17
	PC2		48.74	< 0.001			3	50
	PC3		0.77	0.52			0	0
Lake Mweru *Pseudocrenilabrus* offshore feeders	PC1	5, 28	70.33	< 0.001	6	15	8	53
	PC2		5.84	0.001			2	13
	PC3		26.23	< 0.001			10	66
Lake Mweru *Pseudocrenilabrus* rocky shore dwellers	PC1	2, 21	2.97	0.076	3	3	0	0
	PC2		1.43	0.264			0	0
	PC3		1.54	0.24			0	0
East and South African radiation comparison	PC1	3, 141	13.66	< 0.001	4	6	3	50
	PC2		24.63	< 0.001			3	50
	PC3		6.03	< 0.001			2	66
Within Lake Mweru radiation comparison	PC1	3, 59	39.06	< 0.001	4	6	5	83
	PC2		30.69	< 0.001			5	83
	PC3		3.31	0.027			0	0

Supplementary Table 3: Species names, sampling locations, and Genbank accession numbers of specimens included in the phylogenetic analysis. Samples are sorted by their mitochondrial DNA clade affiliation (see Fig. 4), not by genus. Shaded rows indicate individuals with cyto-nuclear discordance. Numbers in brackets indicate the number of individuals per species sequenced in this study.

species	sample site	accession no.
Lake Mweru Sargochromis (clade I)		
Sargochromis sp. big miller	Lake Mweru, Nchelenge landing site (4 #)	xxx-xxx
Sargochromis sp. yellow face small teeth	Lake Mweru ,Nchelenge landing site (2 #)	xxx-xxx
Sargochromis sp. yellow face big teeth	Lake Mweru, Nchelenge landing site (1 #)	xxx-xxx
Sargochromis sp. red face	Lake Mweru, Pembe Lagoon (3 #)	xxx-xxx
Sargochromis cf. mellandi	Lake Mweru, Pembe Lagoon (2 #)	xxx-xxx
Sargochromis sp. Chief's Pond	Lake Mweru, Chief's Pond (2 #)	xxx-xxx
Sargochromis sp. (unidentified)	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Sargochromis sp. duck snout	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Sargochromis sp. compressed suborbital	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Sargochromis sp. deep body yellowish	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Sargochromis sp. deep short jaws	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Sargochromis sp. thin face green	Lake Mweru, Nchelenge landing site (1 #)	xxx-xxx
Sargochromis sp. (unidentified)	Lake Mweru	AY913893
Sargochromis sp. (unidentified)	Lake Mweru	AY913894
Sargochromis, other origins (clade I)		
Sargochromis mellandi	Lake Bangweulu, Chinsanka (10 #)	xxx-xxx
Sargochromis mellandi	Lake Bangweulu, Samfya (7 #)	xxx-xxx
Sargochromis mellandi	Lake Bangweulu	AY913904
Sargochromis mellandi	Lake Bangweulu	AY913905
Sargochromis mellandi	Chambeshi	AY913895
Pharyngochromis n. sp.	Zimbabwe: Zambesi (Kandahar)	AY913872
Pharyngochromis n. sp.	Zimbabwe: Zambesi at Victoria Falls	AY913880
Pharyngochromis acuticeps	Gadikwe Lagoon, Okavango Delta, Botswana.	AY913882
Pharyngochromis n. sp.	Zimbabwe: Zambesi at Victoria Falls	AY913868
Chetia flaviventris	Roodeplat Dam nr Pretoria	AY913849
Chetia flaviventris	Roodeplat Dam nr Pretoria	AY913914
Sargochromis mortimeri	below Itezhi Ithezi Dam, Kafue River	AY913888
Sargochromis codringtonii	Gadikwe Lagoon, Okavango Delta, Botswana.	AY913852
Sargochromis codringtonii	House Boat Lagoon, Okavango, Botswana.	AY913875
Pharyngochromis acuticeps	Lake Chivero	AY913845
Sargochromis n. sp. 1	Lisikili Lagoon	AY913874
Pharyngochromis acuticeps	Shakawe, Okavango Delta, Botswana.	AY913848
Pharyngochromis acuticeps	Katima Mulilo, Namibia	AY913846
Sargochromis carlottae	Xakanaxa, Moremi, Okavango, Botswana.	AY913879
Sargochromis n.sp. 2	Lisikili Lagoon	AY913886
Sargochromis carlottae	Oddballs Camp, Okavango Delta, Botswana	AY913850
Serranochromis robustus jallae	Shakawe Camp, Okavango, Botswana	AY913864
Pharyngochromis acuticeps	C73 Mwekera Dam, Middle Zambezi	AY913912
Pharyngochromis acuticeps	C74 Mwekera Dam, Middle Zambezi	AY913913
Sargochromis n. sp. 1	Kalembeza/Zambezi junction, Namibia	AY913870
Sargochromis n. sp. 2	Kalembeza/Zambezi junction, Namibia	AY913892
Sargochromis giardi	Oddballs Camp, Okavango Delta, Botswana	AY913865
Sargochromis cf. mortimeri	Upper Zambezi 15/11/12S 22/54/02E	AY913873
Sargochromis sp.	Zimbabwe: Zambezi (Victoria Falls)	AY913878
Sargochromis giardi	Okavango delta	AY913866
Chetia brevicauda	Mozambique: Mussapa River near Zomba	AY913877
Chetia brevicauda	Mozambique: Mussapa River near Zomba	AY913876
Chetia brevicauda	Mussapa River near Zomba	AY913906
Chetia brevicauda	Mussapa River near Zomba	AY913885
Serranochromis macrocephalus	Marienfluss, Namibia	AY913937
Sargochromis cf. giardi	Upper Zambezi 15/11/12S 22/54/02E	AY913909

Sargochromis coulteri	Ruacana, Cunene River, Namibia	AY913887
Sargochromis coulteri	Ruacana, Cunene River, Namibia	AY913891
Sargochromis sp.	Zimbabwe: Zambezi (Victoria Falls)	AY913871
Sargochromis 'giardi' = cf. giardi 2	Zimbabwe: Zambezi (Victoria Falls)	AY913881
Serranochromis macrocephalus	Shakawe Camp, Okavango, Botswana	AY913863
Serranochromis macrocephalus	Shakawe Camp, Okavango, Botswana	AY913907
Serranochromis (clade II)		
Serranochromis macrocephalus	Zimbabwe: Zambezi (Victoria Falls)	AY913920
Serranochromis macrocephalus	Barotse Upper Zambezi River	AY913933
Serranochromis macrocephalus	Lisikili Kalambeza, Namibia	AY913915
Serranochromis macrocephalus	Chanyanya KF	AY913903
Lake Mweru Serranochromis large tooth (clade III)		
Serranochromis sp. macrocephalus-like pop. 1	Lake Mweru, Pembe (10 #)	xxx-xxx
Serranochromis sp. macrocephalus-like pop. 2	Lake Mweru, Isokwe (1 #)	xxx-xxx
Serranochromis sp. macrocephalus-like pop. 3	Lake Mweru, Kabuta (1 #)	xxx-xxx
Serranochromis sp. checkerboard pop. 1	Lake Mweru , Kilwa Island (4 #)	xxx-xxx
Serranochromis sp. checkerboard pop. 2	Lake Mweru, Isokwe Island (1 #)	xxx-xxx
Serranochromis sp. yellow long face pop. 1	Lake Mweru, Kilwa Island (2 #)	xxx-xxx
Serranochromis sp. yellow long face pop. 2	Lake Mweru, Isokwe Island (1 #)	xxx-xxx
Serranochromis sp. long pelvics	Lake Mweru, Isokwe Island (3 #)	xxx-xxx
Serranochromis sp. long face blue	Lake Mweru, Isokwe Island (1 #)	xxx-xxx
Serranochromis cf. stappersi	Lake Mweru, Kilwa Island (1 #)	xxx-xxx
Serranochromis altus / angusticeps (clade III)		
Serranochromis angusticeps pop. 1	Lake Bangweulu, Samfya (2 #)	xxx-xxx
Serranochromis angusticeps pop. 2	Lake Bangweulu, Chinsanka (7 #)	xxx-xxx
Serranochromis angusticeps pop. 3	Lake Mweru, Isokwe Island (2 #)	xxx-xxx
Serranochromis sp. yellow angusticeps	Lake Mweru, Chief's Pond (2 #)	xxx-xxx
Serranochromis sp. checkerboard	Lake Mweru, Kilwa Island (1 #)	xxx-xxx
Serranochromis altus	Lake Bangweulu, Mpanta (1 #)	xxx-xxx
Serranochromis altus	Lake Bangweulu, Mofya (1 #)	xxx-xxx
Serranochromis altus	22 U. Zambezi site 34	AY913911
Serranochromis altus	56 U. Zambezi site 34	AY913918
Serranochromis altus	Barotse Upper Zambezi River	AY913925
Serranochromis robustus robustus	Lake Malawi	AY913932
Serranochromis angusticeps	Lake Bangweulu	AY913926
Serranochromis angusticeps	Lake Bangweulu	AY913930
Serranochromis altus	Shakawe Camp, Okavango, Botswana	AY913919
Serranochromis angusticeps	Lake Bangweulu	AY913896
Serranochromis angusticeps	Lake Bangweulu	AY913924
Serranochromis angusticeps	Lake Bangweulu	AY913928
Serranochromis sp. Mfimbo	Lake Mweru Mfimbo	AY913922
Serranochromis sp. Mfimbo	Lake Mweru Mfimbo	AY913927
Serranochromis sp. Mfimbo	Lake Mweru, Mfimbo	AY913898
Serranochromis angusticeps	Chambeshi	AY913921
Serranochromis angusticeps	Mambova Upper Zambezi River	AY913897
Serranochromis angusticeps	Kandahar, Victoria Falls National Park	AY913862
Serranochromis angusticeps	Zimbabwe: Zambezi (Victoria Falls)	AY913890
Chetia brevis	Driekoppies Dam	AY913854
Chetia brevis	Driekoppies Dam	AY913869
Serranochromis meridianus	RUSI 69447 Limpopo River, Letaba branch	AY913910
Serranochromis meridianus	RUSI 69447 Limpopo River, Letaba branch	AY913889
Serranochromis angusticeps	Ngarange, Okavango River	AY913917
Serranochromis altus	House boat lagoon, near Shakawe, Okavango	AY913902
Serranochromis macrocephalus	Kunene	AY913916
Serranochromis macrocephalus	Seracafena Camp Site, Namibia	AY913936
Lake Mweru Serranochromis small tooth (clade IV)		
Serranochromis sp. diplotaxodon face pop. 1	Lake Mweru, Kilwa Island (4 #)	xxx-xxx
Serranochromis sp. diplotaxodon face pop. 2	Lake Mweru, Isokwe Island (1 #)	xxx-xxx

Serranochromis sp. diplotaxodon face pop. 3	Lake Mweru, Pembe (2 #)	xxx-xxx
Serranochromis sp. diplotaxodon face pop. 4	Lake Mweru, open lake (1 #)	xxx-xxx
Serranochromis sp. stappersi pop. 1	Lake Mweru, open lake (2 #)	xxx-xxx
Serranochromis sp. stappersi pop. 2	Lake Mweru, Pembe Lagoon (3 #)	xxx-xxx
Serranochromis sp. stappersi pop. 3	Lake Mweru, Kilwa Island (1 #)	xxx-xxx
Serranochromis sp. deep red pop. 1	Lake Mweru, Isokwe Island (3 #)	xxx-xxx
Serranochromis sp. deep red pop. 2	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Serranochromis sp. silver long	Lake Mweru, Kalobwa (3 #)	xxx-xxx
Serranochromis sp. dark long pop. 1	Lake Mweru, Isokwe Island (2 #)	xxx-xxx
Serranochromis sp. dark long pop. 2	Lake Mweru, Kilwa Island (2 #)	xxx-xxx
Serranochromis sp. macrocephalus-like	Lake Mweru, Isokwe Island (1 #)	xxx-xxx
Sargochromis cf. mellandi	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Sargochromis sp. red face	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913929
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913938
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913923
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913934
Serranochromis sp. (unidentified)	Lake Mweru	AY226792
Serranochromis stappersi	Lake Mweru, Mwatishi	AY913931
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913929
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913938
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913923
Serranochromis stappersi	Lake Mweru, Mfimbo	AY913925

Serranochromis thumbergi / robustus (clade VI)

Serranochromis thumbergi pop. 1	Lake Bangweulu, Fish Market (2 #)	xxx-xxx
Serranochromis thumbergi pop. 2	Lake Bangweulu, Chinsanka (2 #)	xxx-xxx
Serranochromis robustus	Lake Bangweulu, Chinsanka (4 #)	xxx-xxx
Serranochromis robustus	Lake Mweru, Pembe Lagoon (1 #)	xxx-xxx
Serranochromis sp.	Lake Bangweulu, Mpanta (1 #)	xxx-xxx
Serranochromis thumbergi	Boro River channel, Okavango Delta	AY913844
Serranochromis thumbergi	Wildlife Camp lagoon, Okavango Delta	AY913908
Serranochromis robustus	Chambeshi	AY913899
Serranochromis thumbergi	Lake Bangweulu	AY913900
Serranochromis robustus	Confluence Musola & Kasanka Rivers, CRS	AY913901
Serranochromis robustus	2 Upper Zambezi	AY913867

Thoracochromis (clade V)

Thoracochromis albolabris	Cunene River, Namibia	AY913847
Thoracochromis buysi	Cunene River, Namibia	AY913851
Thoracochromis buysi	Cunene River, Namibia	AY913883
Thoracochromis buysi	Cunene River, Namibia	AY913884

Lower Congo riverine, Orthochromis sp. red fin Lake Mweru (Clade IX)

Thoracochromis demeusii	Lower Congo River between Luozi and Matadi	AY913857
Orthochromis sp. red fin	Lake Mweru, Kabuta (3 #)	Xxx - xxx
Orthochromis polyacanthus	Congo river	AF400712
Schwetzochromis stormsi	Congo river	AY929952
Thoracochromis brauschi	Lake Fwa, Middle Kasai	AY226791
Thoracochromis brauschi	Lake Fwa, Middle Kasai	AY929982
Cyclopharynx fwae	Lake Fwa, Middle Kasai	AY929986
Cyclopharynx fwae	Lake Fwa, Middle Kasai	AF400711

Lake Mweru Pseudocrenilabrus (clade VII)

Pseudocrenilabrus cf. philander	Lake Mweru, Isokwe Island (6 #)	xxx-xxx
Pseudocrenilabrus sp. weed picker small teeth	Lake Mweru, Isokwe Island (2 #)	xxx-xxx
Pseudocrenilabrus sp. weed picker	Lake Mweru, Isokwe Island (8 #)	xxx-xxx
Pseudocrenilabrus sp. telmatochromis pop. 1	Lake Mweru, Isokwe Island (4 #)	xxx-xxx
Pseudocrenilabrus sp. telmatochromis-like pop. 2	Lake Mweru, Kilwa Island (5 #)	xxx-xxx
Pseudocrenilabrus sp. fat broad head	Lake Mweru, Isokwe Island (3 #)	xxx-xxx

Pseudocrenilabrus sp. grey back	Lake Mweru, Isokwe Island (2 #)	xxx-xxx
Pseudocrenilabrus sp. firetail	Lake Mweru, Kalobwa (14 #)	xxx-xxx
Pseudocrenilabrus sp. firetail	Lake Mweru, Kabuta (5 #)	xxx-xxx
Pseudocrenilabrus sp. fire tail black fin	Lake Mweru, Kalobwa (3 #)	xxx-xxx
Pseudocrenilabrus sp. dwarf black pelvic pop. 1	Lake Mweru, Kabuta (2 #)	xxx-xxx
Pseudocrenilabrus sp. dwarf black pelvic pop. 2	Lake Mweru, Kalobwa (3 #)	xxx-xxx
Pseudocrenilabrus sp. broad head black pelvic	Lake Mweru, Kalobwa (9 #)	xxx-xxx
Pseudocrenilabrus sp. long grey	Lake Mweru, Kashikishi beach (2 #)	xxx-xxx
Pseudocrenilabrus sp. yellow white chest	Lake Mweru, Isokwe Island (2 #)	xxx-xxx
Pseudocrenilabrus sp. long brown	Lake Mweru, Kashikishi beach (1 #)	xxx-xxx
Thoracochromis sp. red spotted fin	Lake Mweru (2 #), Kabuta	xxx-xxx
Thoracochromis sp. yellow moeruensis	Lake Mweru (6 #), Kalobwa	xxx-xxx
Thoracochromis sp. grey moeruensis	Lake Mweru (2 #), Isokwe Island	xxx-xxx
Thoracochromis sp. pundamilia-like	Lake Mweru (4 #), Kalobwa	xxx-xxx
Pseudocrenilabrus, other clades		
Pseudocrenilabrus philander	Lake Bangweulu (13 #)	xxx-xxx
Pseudocrenilabrus sp. fire tail black fin	Lake Mweru, Kalobwa (1 #)	xxx-xxx
Pseudocrenilabrus philander	Lake Chilwa	AY913860
Pseudocrenilabrus sp. (misidentified as Schwetzochromis on Genbank)	Ruacana, Cunene River, Namibia	AY913861
Pseudocrenilabrus philander	Zambezi River near Marromeu, Mozambique	AY913859
Pseudocrenilabrus philander	Nkomati	AY913853
Pseudocrenilabrus multicolor	Nile river system	AY929993
Pseudocrenilabrus multicolor	Nile river system	AY930027
Pseudocrenilabrus nicholsi	Lake Ugemba, East Congo	xxx - xxx
Lake Mweru Orthochromis (Clade VIII)		
Orthochromis kalungwishiensis	Kalungwishi River, Lake Mweru (1 #)	xxx - xxx
O. sp. "red cheek"	Lake Mweru, Kalobwa (3 #)	xxx - xxx
Lake Victoria clade		
Astatotilapia velifer	Lake Nabugabo (Victoria)	AF213553
Astatotilapia velifer	Lake Nabugabo (Victoria)	AF213551
Paralabidochromis chilotes	Lake Victoria	AF213540
Neochromis nigricans	Lake Victoria	AF213545
Ptyochromis xenognathus	Lake Victoria	AF213534
Yssichromis laparogramma	Lake Victoria	AF213521
Paralabidochromis plagiodon	Lake Victoria	AF213548
Psammochromis graueri	Lake Kivu	AY226656
Lake Malawi clade		
Protomelas taeniolatus	Lake Malawi	AY913942
Metriaclima callainos	Lake Malawi	AF213620
Copadichromis virginalis	Lake Malawi	AF298943
Astatotilapia calliptera	Lake Malawi	AY913939
Labeothropheus trewavasae	Lake Malawi	AY911790
Rhamphochromis esox	Lake Malawi	AF298913
Lake Tanganyika haplochromine clade		
Pseudosimochromis curvifrons	Lake Tanganyika	AF400735
Gnathochromis pfefferi	Lake Tanganyika	AF400727
Petrochromis orthognathus	Lake Tanganyika	AF400734
Simochromis babaulti	Lake Tanganyika	AF400736
Lobochilotes labiatus	Lake Tanganyika	AF400733
Tropheus polli	Lake Tanganyika	AY929971
East African rivers and lakes		
Astatotilapia burtoni	Lake Tanganyika drainage	AF298905
Astatotilapia burtoni	Lake Tanganyika drainage	AF30000
Haplochromis gracilior	Lake Kivu	AY226790
Haplochromis gracilior	Lake Kivu	AY226788
Astatotilapia paludinosa	Lower Malagarasi River and swamps	AY929994
Astatotilapia bloyeti	Tanzanian/Indian Ocean Rivers	AY929953

Astatotilapia tweddelei	Lake Chilwa	xxx-xxx
Astatoreochromis alluaudi	Lake Victoria and Lake Edward region	AY929996
Astatoreochromis alluaudi	Lake Victoria and Lake Edward region	AY929966
Thoracochromis pharyngalis	Lake Edward	xxx
Thoracochromis pharyngalis	Lake Edward	xxx

Orthochromis Lake Tanganyika drainage

Orthochromis malagaraziensis	Lake Tanganyika drainage (upper Malagarazi, Burundi)	AY929951
Orthochromis malagaraziensis	Lake Tanganyika drainage (upper Malagarazi, Burundi)	AY929949
Orthochromis malagaraziensis	Lake Tanganyika drainage (upper Malagarazi, Burundi)	AF400714
Orthochromis mazimeroensis	Lake Tanganyika drainage (upper Malagarazi, Mazimero and Nanganga rivers)	AY929948
Orthochromis mazimeroensis	Lake Tanganyika drainage (upper Malagarazi, Mazimero and Nanganga rivers)	AY930032
Orthochromis mazimeroensis	Lake Tanganyika drainage (upper Malagarazi, Mazimero and Nanganga rivers)	AF400715
Orthochromis mosoensis	Lake Tanganyika drainage (upper Malagarasi, Burundi)	AY929950
Orthochromis mosoensis	Lake Tanganyika drainage (upper Malagarasi, Burundi)	AY930033
Orthochromis kasuluensis	Lake Tanganyika drainage (Malagarazi, upper Ruchugi drainage near Kasulu)	AY930031
Orthochromis kasuluensis	Lake Tanganyika drainage (Malagarazi, upper Ruchugi drainage near Kasulu)	AY929944
Orthochromis rugufuensis	Lake Tanganyika drainage (upper Rugufu system, western Tanzania)	AY929945
Orthochromis rubrolabialis	Lake Tanganyika drainage (Malagarazi, Majamazi and Ugalla rivers)	AY929946
Orthochromis uvinzae	Lake Tanganyika drainage (middle Malagarasi drainage at Uvinza, Tanzania)	AY929943
Orthochromis luichensis	Lake Tanganyika drainage (Luiche River, western Tanzania)	AY929947

Lamprologini

Lamprologus congoensis	Congo River at Matadi, Stanley Pool, Stanley Falls and Upper Congo	AF400719
Lamprologus mocquardi	Congo River and upper Ubangi.	AF400720
Lamprologus callipterus	Lake Tanganyika	AF400718
Neolamprologus brichardi	Lake Tanganyika	AF400721
Neolamprologus toae	Lake Tanganyika	AF400723
Julidochromis marlieri	Lake Tanganyika	AF400717

Limnochromis, Eretmodus and outgroups

Limnochromis auritus	Lake Tanganyika	AF400728
Eretmodus cyanostictus	Lake Tanganyika	AF400707
Bathybates sp.	Lake Tanganyika	U12556
Oreochromis tanganicae	Lake Tanganyika	AY929940

Supplementary Table 4: Species names and sampling locations of the specimens included in the morphological analysis. References and accessions are given where available. Sample sizes are only given for the species measured in this study.

species	n	lake	collection site	reference
Lake Mweru Sargochromis radiation				
Sargochromis sp. big miller	7	Mweru	Nchelenge landing site	Stelkens et al, this study
Sargochromis sp. yellow face small teeth	4	Mweru	Nchelenge landing site	Stelkens et al, this study
Sargochromis sp. yellow face big teeth	4	Mweru	Nchelenge landing site	Stelkens et al, this study
Sargochromis sp. red face	7	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis cf. mellandi	6	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. Chief's Pond	3	Mweru	Chief's Pond	Stelkens et al, this study
Sargochromis sp. Pembe	4	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. duck snout	2	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. compressed suborbital	1	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. deep body yellowish	1	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. deep short jaws	1	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. short blunt head	1	Mweru	Pembe Lagoon	Stelkens et al, this study
Sargochromis sp. thin face green	1	Mweru	Nchelenge landing site	Stelkens et al, this study
Lake Mweru Serranochromis large tooth radiation				
Serranochromis cf. macrocephalus pop. 1	8	Mweru	Pembe Lagoon	Stelkens et al, this study
Serranochromis cf. macrocephalus pop. 2	3	Mweru	Pembe Lagoon	Stelkens et al, this study
Serranochromis cf. macrocephalus pop. 3	3	Mweru	Kabuta (rocky shore, angling)	Stelkens et al, this study
Serranochromis sp. checkerboard	5	Mweru	Kilwa Island (rocky outcrops)	Stelkens et al, this study
Serranochromis sp. yellow long face pop. 1	6	Mweru	Kilwa/Isokwe (offshore)	Stelkens et al, this study
Serranochromis sp. yellow long face pop. 2	6	Mweru	Nchelenge landing site	Stelkens et al, this study
Serranochromis sp. long pelvics	5	Mweru	Nchelenge landing site	Stelkens et al, this study
Serranochromis sp. long face blue	1	Mweru	Nchelenge landing site	Stelkens et al, this study
Lake Mweru Serranochromis small tooth radiation				
Serranochromis sp. diplotaxodon face pop. 1	4	Mweru	Kilwa/Isokwe (offshore)	Stelkens et al, this study
Serranochromis sp. diplotaxodon face pop. 2	3	Mweru	Nchelenge landing site	Stelkens et al, this study
Serranochromis sp. diplotaxodon face pop. 3	3	Mweru	Pembe Lagoon	Stelkens et al, this study
Serranochromis sp. diplotaxodon face pop. 4	2	Mweru	Kilwa/Isokwe (offshore)	Stelkens et al, this study
Serranochromis stappersi pop. 1	3	Mweru	Kilwa/Isokwe (offshore)	Stelkens et al, this study
Serranochromis stappersi pop. 2	3	Mweru	Pembe Lagoon	Stelkens et al, this study
Serranochromis stappersi pop. 3	2	Mweru	Kilwa Island (rocky outcrops)	Stelkens et al, this study
Serranochromis sp. deep red pop. 1	6	Mweru	Nchelenge landing site	Stelkens et al, this study
Serranochromis sp. deep red pop. 2	2	Mweru	Pembe Lagoon	Stelkens et al, this study
Serranochromis sp. silver long	6	Mweru	Kalobwa (offshore)	Stelkens et al, this study
Serranochromis sp. dark long pop. 1	2	Mweru	Nchelenge landing site	Stelkens et al, this study
Serranochromis sp. dark long pop. 2	3	Mweru	Kilwa/Isokwe (offshore)	Stelkens et al, this study
Lake Mweru Pseudocrenilabrus radiation				
Pseudocrenilabrus cf. philander	6	Mweru	Isokwe Island	Stelkens et al, this study
Pseudocrenilabrus sp. weed picker small teeth	5	Mweru	Isokwe Island	Stelkens et al, this study
Pseudocrenilabrus sp. weed picker	7	Mweru	Isokwe Island	Stelkens et al, this study
Pseudocrenilabrus sp. telmatochromis-like pop. 1	6	Mweru	Isokwe Island	Stelkens et al, this study
Pseudocrenilabrus sp. telmatochromis-like pop. 2	2	Mweru	Kilwa Island (rocky outcrops)	Stelkens et al, this study
Pseudocrenilabrus sp. grey back	6	Mweru	Isokwe Island	Stelkens et al, this study
Pseudocrenilabrus sp. firetail	8	Mweru	Kalobwa (beach seine)	Stelkens et al, this study

Pseudocrenilabrus sp. firetail	4	Mweru	Kabuta (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. fire tail black fin	3	Mweru	Kalobwa (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. dwarf black pelvic pop. 1	4	Mweru	Kabuta (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. dwarf black pelvic pop. 2	1	Mweru	Kalobwa (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. broad head black pelvic	3	Mweru	Kalobwa (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. long grey	2	Mweru	Isokwe Island (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. pale orange	1	Mweru	Isokwe Island (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. yellow white chest	2	Mweru	Isokwe Island (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. long brown	1	Mweru	Kashikishi (beach seine)	Stelkens et al, this study
Pseudocrenilabrus sp. pale deep	2	Mweru	Kashikishi (beach seine)	Stelkens et al, this study
Thoracochromis sp. red spotted fin	7	Mweru	Kabuta (rocky shore, angling)	Stelkens et al, this study
Thoracochromis sp. black moeruensis	1	Mweru	Kalobwa (rocky shore)	Stelkens et al, this study
Thoracochromis sp. yellow moeruensis	12	Mweru	Kalobwa (rocky shore)	Stelkens et al, this study
Thoracochromis sp. grey moeruensis	2	Mweru	Isokwe Island (beach seine)	Stelkens et al, this study
Thoracochromis sp. pundamilia-like	3	Mweru	Kabuta (rocky shore, angling)	Stelkens et al, this study

Serranochromis other

Serranochromis thumbergi pop. 1	1	Bangweulu	Fish Market in Samfya	Stelkens et al, this study
Serranochromis thumbergi pop. 2	5	Bangweulu	Chinsanka fish landing	Stelkens et al, this study
Serranochromis robustus	1	Mweru	Pembe Lagoon	Stelkens et al, this study
Serranochromis angusticeps pop. 1	1	Mweru	Chief's Pond	Stelkens et al, this study
Serranochromis angusticeps pop. 2	3	Mweru	Nchelenge fish landing	Stelkens et al, this study
Serranochromis angusticeps pop. 3	4	Bangweulu	Chinsanka fish landing	Stelkens et al, this study

Lake Makgadikgadi radiation

Thoracochromis albolabris	LMkg	-	SAIAB 41076
Thoracochromis buysi	LMkg	-	SAIAB 63330
Chetia brevicauda	LMkg	-	SAIAB 67651
Chetia brevis	LMkg	-	SAIAB 69426
Chetia flaviventris	LMkg	-	SAIAB 53629 & 61095
Pharyngochromis acuticeps pop. 1	LMkg	-	SAIAB 44038
Pharyngochromis acuticeps pop. 2	LMkg	-	SAIAB 52229 & 21293
Pharyngochromis sp.	LMkg	-	Unaccessioned
Sargochromis carlottae	LMkg	-	SAIAB 52433 & 26853
Sargochromis codringtonii	LMkg	-	SAIAB 52481
Sargochromis coulteri	LMkg	-	SAIAB 39104 & 42033
Sargochromis giardi	LMkg	-	SAIAB 68770 & 22226
Sargochromis giardi cf.	LMkg	-	SAIAB 69445
Sargochromis mortimeri cf.	LMkg	-	SAIAB 69446
Sargochromis sp. 'dish'	LMkg	-	SAIAB 63433
Serranochromis altus cf.	LMkg	-	SAIAB 28709
Serranochromis angusticeps	LMkg	-	SAIAB 29314
Sargochromis mellandi	LMkg	-	Unaccessioned
Serranochromis meridianus	LMkg	-	SAIAB 57265 & 57266
Serranochromis macrocephalus Cunene	LMkg	-	SAIAB 63276
Serranochromis macrocephalus Okavango	LMkg	-	SAIAB 23318
Serranochromis robustus (jallae?)	LMkg	-	SAIAB 63276
Serranochromis robustus robustus	LMkg	-	SAIAB 23318
Serranochromis thumbergi	LMkg	-	SAIAB 52506
Serranochromis macrocephalus Congo	LMkg	-	SAIAB 50055
Serranochromis stappersi	LMkg	-	SAIAB 20984

Lake Victoria radiation

Pundamilia pundamilia	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129 -228 (1998)
Pundamilia Luanso	LV	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Neochromis greenwoodi	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129 -228 (1998)

Neochromis rufocaudalis	LV	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Pyochromis xenognathus	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129
Macropleurodus bicolor	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129
"Haplochromis" nubilus	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
"Haplochromis" velifer	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Lipochromis obesus	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Harpagochromis guiarti	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Harpagochromis paraguiarti	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Prognathochromis longirostris	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Prognathochromis dentex	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Harpagochromis cavifrons	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Pyxichromis orthostoma	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Labrochromis teegelaari	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Labrochromis mylergates	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Mbipia mbipi	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129
Neochromis omnicaeruleus	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129
Pundamilia nyererei	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129
Labrochromis ishmaeli	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Ptyochromis sauvagei	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Harpagochromis orange rock hunter	LV	-	Seehausen et al. Ichthyol. Explor. Freshwat. 9, 129 -228 (1998)
Yssichromis laparogramma	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Yssichromis piceatus	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Platytaeniodus degeni	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
"Haplochromis" elegans	LV	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996

Lake Malawi clade

Copadichromis virginalis	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Rhamphochromis esox	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Diplotaxodon limnothrissa	LM	-	Eccles, D. H. & Trewavas, Lake Fish Movies, Herten, Germany, 1989
Taeniolethrinops laticeps	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980

Ps. tropheops "olive"	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Ps. tropheops "mauve" Nkatha	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Ps. tropheops "mauve" Mara	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Metriaclima fainzilberi "Ruarwe"	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Metriaclima fainzilberi "Mara"	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Metriaclima zebra "Nkhata"	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Metriaclima livingstonii	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Mylochromis sphaerodon	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Trematocranus placodon	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Hemitaeniochromis urotaenia	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Caprichromis orthognathus	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Protomelas spilopterus	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Buccochromis lepturus	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Nimbochromis polystigma	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Dimidichromis compressiceps	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Nimbochromis linni	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Alticorpus mentale	LM	-	Greenwood, P. H. Krauss Intl., Munich, 1980
Rhamphochromis lucius	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Rhamphochromis ferox	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Rhamphochromis leptosoma	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Rhamphochromis longiceps	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Rhamphochromis macrophthalmus	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Rhamphochromis brevis	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Rhamphochromis woodi	LM	-	Joyce et al. Nature, 435, 5, 90-95 (2005)

Other East and North African haplochromines

Pseudocrenilabrus philander	Lake Bangweulu	-	Stelkens et al, this study
Astatoreochromis alluaudi	LV and LE region	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Thoracochromis petronius	Lake Edward	-	Seehausen, O. Verduijn Cichlids, Zevenhuizen, Netherlands, 1996
Thoracochromis pharyngalis	Lake Edward	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Thoracochromis brauschii	Lake Fwa	-	Turner, G. F., J. Fish Biol. 44, 799–807 (1994)
Astatotilapia flavijosephi	Lake Kinneret	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Astatotilapia bloyeti	East African rivers	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Astatotilapia sparsidens	Lake Manyara	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Astatotilapia burtoni	LT and rivers and lakes	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Astatotilapia calliptera	LM region	-	Joyce et al. Nature, 435, 5, 90-95 (2005)
Astatotilapia sp. Chilwa	Lake Chilwa	-	Joyce et al. Nature, 435, 5, 90-95 (2005)

Chapter 6

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

Rike B. Stelkens[1,2,3] *, Ole Seehausen[1, 2]

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1. Division of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, Baltzerstr. 6, CH-3012 Bern, Switzerland
2. Department of Fish Ecology and Evolution, Centre of Ecology, Evolution and Biogeochemistry (CEEB), Eawag Swiss Federal Institute of Aquatic Science and Technology, Seestrasse 79, CH-6047 Kastanienbaum, Switzerland
3. Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland

* Corresponding author: email: rike.stelkens@unil.ch; tel: +41 21 692 4249; fax: +41 21 692 4165

ABSTRACT

The notion 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 populations 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 same 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 due to diversion of drainage systems.

KEYWORDS: assortative mating, ecological speciation, hybridization, Lake Mweru, mate choice, premating isolation, *Pseudocrenilabrus*.

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 postzygotic isolation builds up as a byproduct of the genetic differences accumulating in diverging populations due to drift (Gavrilets and Boake 1998) and selection (Dobzhansky 1951; Coyne and Orr 2004). Classical theory of reinforcement (Dobzhansky 1951; Noor 1995; Noor 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 and 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 and Schluter 2006), through divergent selection directly acting on (female) mating preferences, e.g. when the conspicuousness of a (male) sexual trait varies with the signalling environment (Endler 1992; Schluter and 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 and Kondrashov 1999; Doebeli and 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 and Via 2000; Jiggins et al. 2001; Nosil and 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 and Willis 2007). One prediction of the ecological speciation hypothesis is that in systems in which phenotypic and genetic divergence vary independently, phenotypic distance is the stronger predictor of assortative mating (McPeek and 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/Democratic Republic of the Congo) (Fig. 1). 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.

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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*, *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 Koblmüller 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.

Fig. 1: 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.

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 (Stelkens et al. in prep.). 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 prep.). 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.
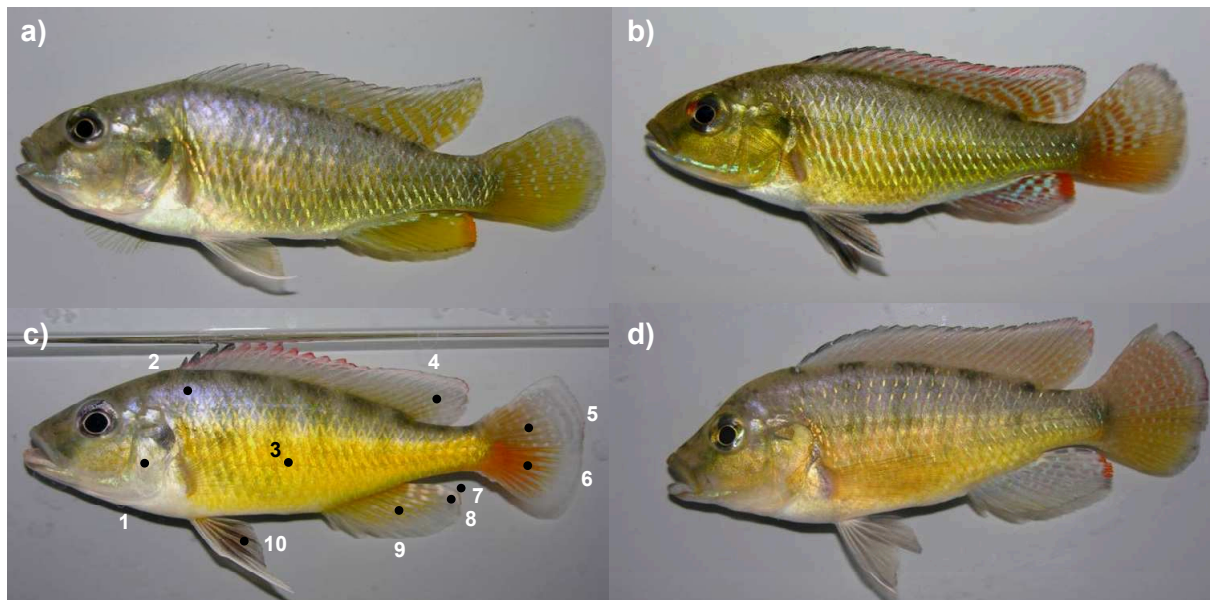
Figure 2: Males of the four populations tested in mate choice experiments; a) *P. philander* from Lake Bangweulu, a distantly related generalist that colonized Lake Mweru. b) *P. philander* from Lake Mweru, a generalist representing the second lineage colonizing Lake Mweru. c) *P. sp. grey back*, a closely related, but ecologically derived type from Lake Mweru. d) *P. sp. weed picker,* another closely related, but ecologically derived type from Lake Mweru. b), c) and d) are sympatric, while a) is allopatric to all. On c) the position of all ten landmarks used for visual scoring of male colouration is indicated.

We conducted mate choice experiments in the laboratory using four different populations: 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 2 a); 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 2 b); iii) a sympatric *P. sp. 'grey back'* population from Lake Mweru, representing an ecologically derived phenotype (Fig 2 c); iv) a sympatric *P. sp. 'weed picker'* population from Lake Mweru (Fig 2 d), representing another ecologically derived phenotype with a different ecological specialization than (c). The three members of the

adaptive radiation (ii, iii, 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, iv). Then we tested the two allopatric *P. philander* against each other (i versus 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.

METHODS

Experimental animals
The four populations were collected in Lakes Mweru and Bangweulu in September 2005. 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. 1) (Jackson 1961). Although the linear distance between the lakes is only 250 km, the length of the connecting river is ca 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. 2a and 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 characterised 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 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
P. sp. 'grey back' and *P. sp.* 'weed picker' occur in sympatry with *P. philander* in Lake Mweru, but they differ strikingly in morphology and colouration (Fig. 2c and d). *P. 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.

P. 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 populations (*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 x 40 x 40 cm large aquaria, divided into three equally sized compartments. To measure female mating preferences based on actual spawning events we used the 'egg catcher' experimental design described in Kidd et al. (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 (Fig. 3). The walls were perforated with holes of 0.5cm diameter every 2 cm^2, allowing females to assess the full sensory repertoire of male mating cues, including chemical (Plenderleith et al. 2005) and visual cues (Seehausen and 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 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.
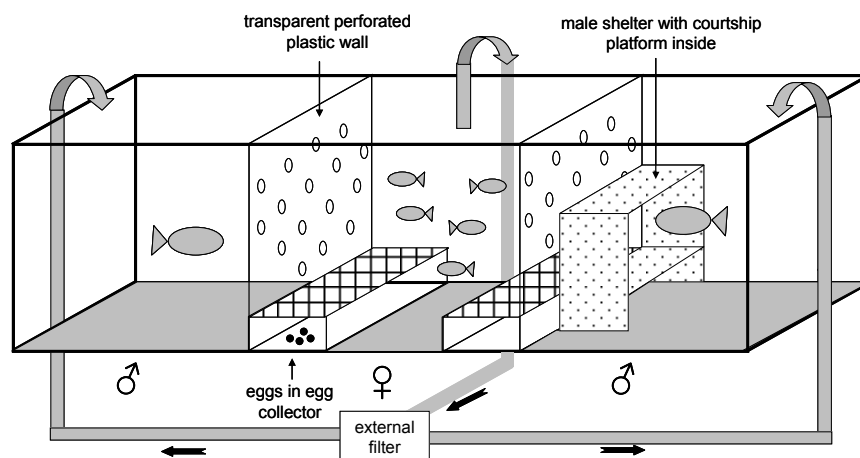
Fig. 3: Experimental design. Egg collectors were perforated with holes big enough to allow eggs to fall through during spawning. Only one spawning shelter is shown for illustrative purposes, but shelters were present in both male compartments. The direction of water flow is indicated by arrows.

Water circulation in the tank was manipulated such that water constantly flew 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 versus *grey back*; Experiment 2) *philander* Mweru versus *weed picker*. The third experiment tested for assortative mating between the two derived members of the endemic radiation: Experiment 3) *grey back* versus *weed picker*. The fourth experiment tested for assortative mating between the allopatric populations of the ancestral phenotype: Experiment 4) *philander* Mweru versus *philander* Bangweulu.

All males and females used in this study were first generation offspring (F1) of wild caught fish, 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 two days before one male was put into each of the adjacent compartments. Females were introduced in groups of 3-14 individuals to facilitate behavioural acclimatisation with minimum levels of aggression. Only females of one of the two species tested were used per trial. The position of male types in the aquarium was randomised over trials. Egg collectors were checked at least three times a day (morning, noon, and evening) for eggs. 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.

Only spawnings of females from different families were considered genetically independent data points. Hence females were introduced to the experimental tank in sib groups. As soon as one female had spawned, we removed all others and replaced them against another sib group. In experiment 2, where only wild caught males and females of unknown pedigree were used, every female was allowed to spawn once and all spawnings were treated as genetically independent data points. 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. 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. Since we were interested in population level divergence in mate choice, this should not bias our test results.

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.

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 (Fig. 4).

Paired t-tests were applied to see if body size differed between the males of the two species used in each experiment. Further, we assigned males to categories of 'winners' and 'losers', depending on which of the two obtained more eggs, and then 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 populations, isolation indices were calculated using $I_{trial} = (E_{con} - E_{hetero})/E_{total}$ where $E_{con} - E_{hetero}$ 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 ($I_{exp1-4\_female\ type}$). 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 population comparisons (I_{exp1-4}), we then averaged over the two female type-specific isolation indices.

Analysis of genetic distance

To compare genetic distances between the four populations 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 population (Mendelson 2003; Chapman and Burke 2007). All distances were corrected for within-population variation among haplotypes by subtracting mean within-population distances from mean pairwise between-population distances (Nei 1987; Mendelson 2003) (Table 1b).

Analysis of ecomorphological distance

To quantify ecomorphological variation within and among the four populations, we calculated phenotypic distances from 13 linear morphological characters measured with digital callipers to the nearest 0.01 millimetre on between 13 and 18 individuals per population (Table 1c). 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 cichlids and other fishes (Caldecutt and Adams 1998; Ruber and Adams 2001; Hendry et al. 2002; Kassam et al. 2003; Clabaut et al. 2007). The 13 morphological characters that we chose are known to reflect subtle variation in resource use of haplochromine cichlids (Caldecutt and Adams 1998; Ruber and 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 body size between populations (one-way ANOVA: $F_{3,61} = 2.99$, $p = 0.038$). A Tukey-Kramer test showed that *weed picker* were on average larger (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.

Due to the size differences between types, we applied statistical corrections following methods described in Hendry et al. (2001). First, linear regressions were calculated within populations for each of the traits against either standard length (in case of BD, HL) or head length (in case of the remaining nine traits). These population-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 populations 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-population 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 population based on the adjusted, morphological traits (M_{std}) alone. This method calculates the distance of each individual to the multivariate population 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 populations was estimated by calculating P_{st} values (the phenotypic surrogate for Qst (Spitze 1993)) for each population 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 populations we visually scored the colour of males in breeding dress at 10 different places on body and fins (Fig. 2c). At every landmark, colour was scored as either translucent, white, grey, black, blue, green, yellow, orange, or red. To convert the qualitative score data into a distance metric we used the 'simple matching coefficient' (SMC) (Digby and 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 population (Table 1d). All colour distances were corrected for within-population 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, 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 three-factorial ANOVA with egg number per male as dependent variable, using a generalized linear model with binomial distribution and logit link function. 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 would be confirmed by a significant interaction term.

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 versus genetic distance, we performed a three-factorial ANOVA on egg numbers with 'female origin' and 'male origin' (either Mweru or Bangweulu) and the interaction term between them (female origin * male origin) as factors. Here, the null hypothesis assumes assortative mating, hence the non-significance 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. All analyses were performed in JMP Professional 6.0.0 (SAS Institute).

RESULTS

Mate choice experiments

In Experiment 1, 10 spawnings were obtained from each population (*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 ($t = 2.14$, $p = 0.031$; all p-values one-tailed, Fig. 4a). *Grey back* females on the other hand did not mate significantly assortatively ($t = -0.86$, $p = 0.205$). Overall there was significant assortative mating in this species pair ($t = -2.0$, $p = 0.030$). 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 two-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 population (*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 *grey back* 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 ($t = -0.58$, $p = 0.285$; Fig. 4b), *philander* Mweru females again mated strongly assortatively ($t = -3.89$, $p = 0.001$). Overall there was a strong trend for assortative mating in this species pair ($t = 1.59$, $p = 0.061$). 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 ($t = 2.14$, $p = 0.043$) and a strong assortative trend was observed for *weed picker* females ($t = -$

1.69, $p = 0.055$; Fig. 4c). Overall there was significant assortative mating in this species pair ($t = -2.79$, $p = 0.010$). 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 strong trend for *weed picker* males to be more successful with *weed picker* females ($Z = -1.22$, $p = 0.116$).
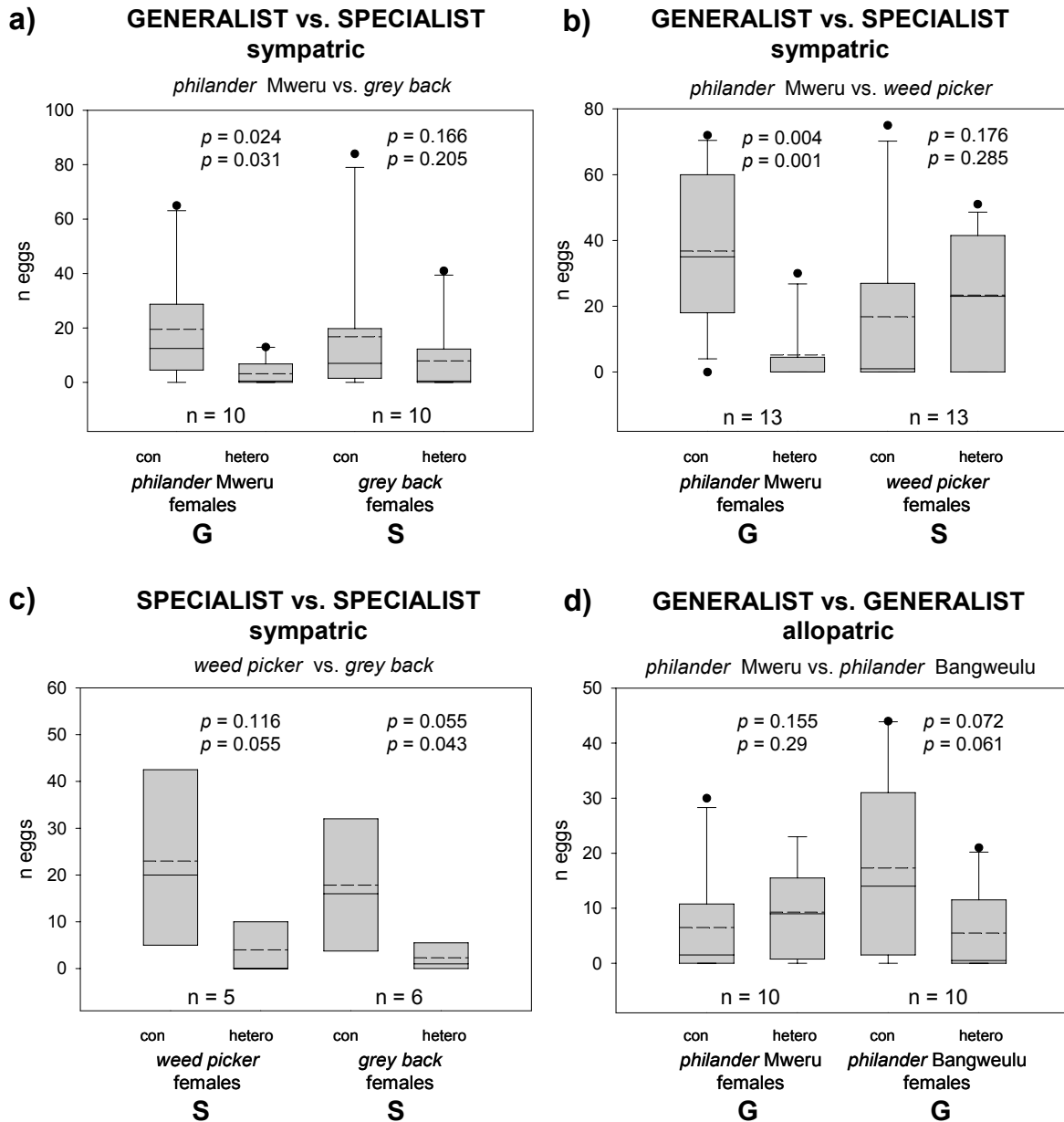

Fig. 4: Results of mate choice experiments between four populations of *Pseudocrenilabrus* with varying degrees of genetic and phenotypic divergence. *Philander* Mweru females mated assortatively against both closely related, sympatric types (a, b), but not against males from the much more distantly related, but phenotypically similar allopatric population (d). The two genetically highly 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, 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.

In Experiment 4, 10 spawnings were obtained from each population (*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: $t = 1.7$, $p = 0.061$; *philander* Mweru females: $t = 0.58$, $p = 0.29$; 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 ($Z = -1.01$, $p = 0.155$).

Table 1: a) Premating isolation indices for all four populations 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. The number of individuals per population is shown in brackets.

a)

Premating isolation	*philander* Bangweulu	*philander* Mweru	*weed picker* Mweru	*grey back* Mweru
philander Bangweulu	-	0.061	-	-
philander Mweru		-	0.227	0.404
weed picker Mweru			-	0.401
grey back Mweru				-

b)

Genetic distance	*philander* Bangweulu	*philander* Mweru	*weed picker* Mweru	*grey back* Mweru
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	*philander* Mweru	*weed picker* Mweru	*grey back* Mweru
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	*philander* Mweru	*weed picker* Mweru	*grey back* Mweru
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)				-

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 ($I_{exp2\_philander \, Mweru} = 0.228$; $I_{exp2\_weed \, picker} = -0.237$), 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 versus the distantly related allopatric population (Experiment 4), only Bangweulu females preferred males of the 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
Body size has been shown to be important for mate choice in cichlids (Seehausen and Van Alphen 1998; Schliewen et al. 2001; van Breukelen and Draud 2005; Gerlai 2007) and other fish (Reynolds and Gross 1992; Nagel and Schluter 1998; Hendry et al. 2000) but in our experiments 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 populations from Lake Mweru (distances between all four populations are summarized in Table 1b).

Ecomorphological divergence
Significant between-population 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). 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 confirms that the two populations have similar morphological adaptations and inhabit similar ecological niches in the two different lakes.

Grey backs were significantly more elongate (smaller body depth) and had narrower snouts than all other populations. They also had a shorter but wider lower jaw, and larger eyes than all other populations. 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 populations. 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. There were significant differences between populations, 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 clearly 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 populations, 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 clearly different from sympatric *philander* Mweru along PC3. On the contrary, *philander* Bangweulu and *philander* Mweru broadly overlapped in morphospace along all axes.
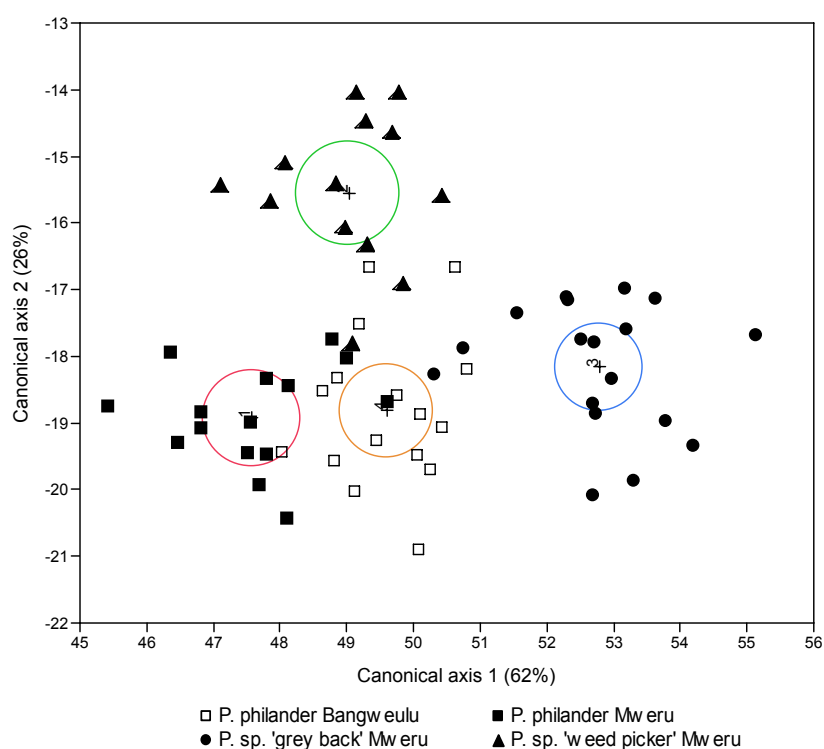
Fig. 5: Canonical discriminate analysis based on 13 morphological traits measured on individuals from the four different populations. All but seven individuals were assigned correctly to their source population. Circles represent 95 % confidence interval spheres around multivariate group means (indicated by +).

The canonical discriminant analysis correctly assigned all but seven out of 62 individuals to the four source populations (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). This was followed by the phenotypic distances between the sympatric generalist, *philander* Mweru and both derived specialists (*philander* Mweru versus *grey back* Mweru: P_{st} = 0.434; *philander* Mweru versus *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 versus *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; all Pst-test results are summarized in Table 1c).

In summary, the largest ecomorphological distances were found among the three sympatric Lake Mweru populations, while the allopatric *philander* Lake Bangweulu was intermediate between all three Lake Mweru species, and most similar to the *philander* Mweru.

Divergence in male colouration
Canonical discriminant analysis revealed that the three sympatric species from Lake Mweru have completely non-overlapping 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 1 out of 35 individuals were correctly assigned to the four source populations (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 simple matching coefficients (SMC), highest dissimilarity in colouration was found between the two sympatric ecological specialists from Lake Mweru, *weed picker* and *grey back* (SMC = 0.607). Colour differences between the two ecologically derived species and the two generalists were small to moderately strong (*philander* Bangweulu versus *grey back*: SMC = 0.546; *philander* Bangweulu versus *weed picker*: SMC = 0.236; *philander* Mweru versus *grey back*: SMC = 0.391; *philander* Mweru versus *weed picker*: SMC = 0.346), while the two populations of ancestral phenotype *philander* Mweru and *philander* Bangweulu were rather similar also in colouration (SMC = 0.379).; all SMC-test results are summarized in Table 1d).

In summary, largest colour dissimilarities were found among the three sympatric species from Lake Mweru, whereas the allopatric *philander* populations were rather similar in phenotype. Remarkably, the dissimilarities in male colour closely resembled those in ecomorphology.
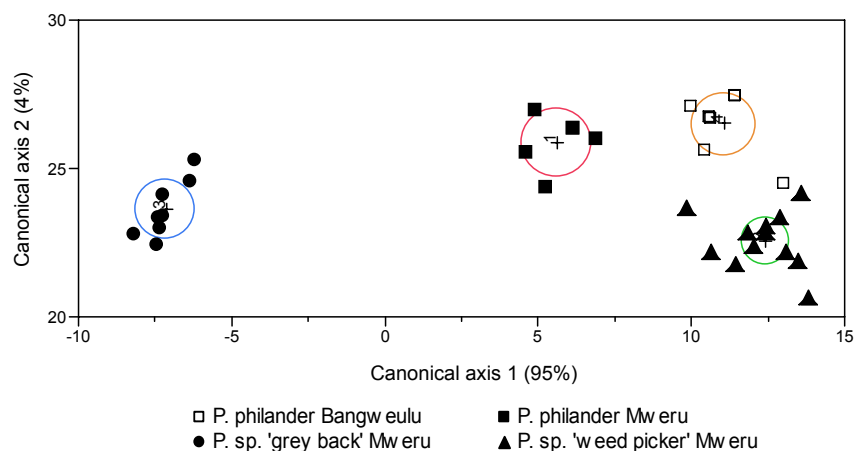
Fig. 6: Canonical discriminate analysis based on male colouration scores taken on 10 morphological landmarks. All but one individual were assigned correctly to their source population. Circles represent 95 % confidence interval spheres around multivariate group means (indicated by +).

Predictors of premating isolation

Phenotypic distance was a good predictor of premating isolation (Fig. 7). The greater the ecomorphological distance between populations, 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 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 (experiment 1-3) the interaction between male and female species was significant ($Chi^2_{3,114} = 18.96$, 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 ($Chi^2_{3,40} = 4.0$, p (model) $= 0.261$, p (interaction) $= 0.684$) confirming our prediction that population origin (Lake Mweru or Bangweulu) did not play a role in mating decisions.
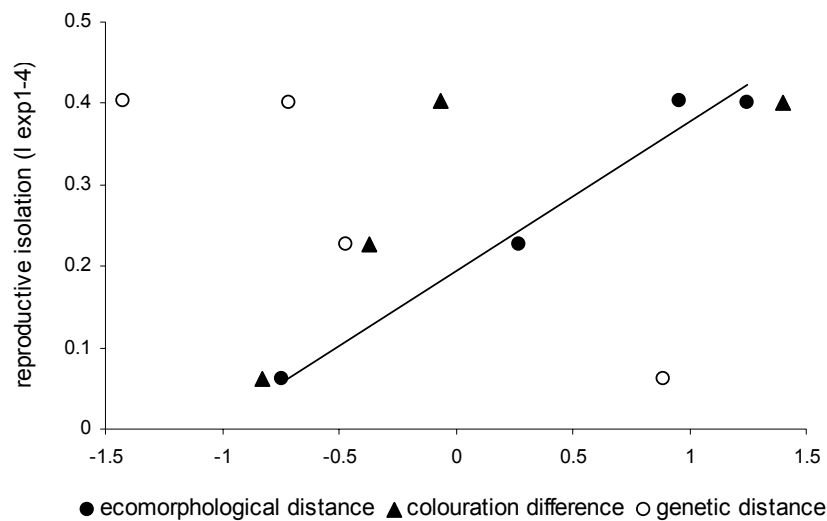
Fig. 7: Testing genetic and phenotypic distance as predictors of premating isolation (represented by the isolation index **I** $_{\text{exp1-4}}$) between the four populations. Isolation increased with ecomorphological distance (significantly, regression line shown) and with colouration differences (non-significantly), 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 populations 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.

Genetic distance between populations 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: $Chi^2_{1,154} = 0.18$, $p = 0.668$), 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 ($Chi^2_{4,154}$ = 20.13, p (model) < 0.001, p (identity) < 0.001, p (genetic distance) = 0.565).

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 and 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 and 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 and 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 populations 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, *P. sp. weed picker*, inhabits the densely vegetated riparian zone and is an ecologically derived specialist, feeding on insect larvae. The third, *P. 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 (Fig. 5 and 6). At the same time, the two allopatric, generalist populations were most 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 closest 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.

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, $\rho = 0.83$, $p = 0.042$). These results are consistent with ecological speciation. 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 and 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 to 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 postzygotic isolation barriers among species in other cichlid radiations with comparably recent divergence times (Crapon de Caprona and 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 versus ecological reinforcement would involve mate choice trials between ecologically divergent populations that have no history of sympatry (Hollander et al. 2005; Vines and Schluter 2006). If for instance, *P. philander* Bangweulu females mated at random when choosing between males of their own population and males of the phenotypically divergent

species from Lake Mweru (*grey back, weed picker*), 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.

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Chapter 7

Conclusions and Synthesis

In this final chapter I summarize the main findings of the work presented in this thesis. I discuss their relevance for our understanding of interspecific hybridization in adaptive evolution and indicate how my results might guide future research.

Hybrid fitness and determining the time window for successful hybridization

A long known pattern in evolutionary biology is that the strength of reproductive isolation between species increases with the amount of time the species have evolved independently from one another (Coyne and Orr 1989, 1997). Isolation is caused by behavioural incompatibilities between males and females affecting the likelihood of heterospecific matings and/or by accumulating Dobzhansky-Muller genetic incompatibilities negatively affecting hybrid fertility or viability (Dobzhansky 1936; Muller 1942; Lynch 1991; Turelli and Orr 2000). Over the last 20 years, various taxa have been investigated for the rate at which pre- and postmating compatibilities decay with divergence time, and it became quickly apparent that some species groups show surprisingly similar decay rates, while others vastly differ in the amount of time that passes before successful hybridization becomes impossible (reviewed in **Chapter 4**).

However, certain rules may be widely applicable. Premating and postmating isolation are thought to accumulate at similar rates when species are allopatric because genetic drift should affect all traits equally, regardless of whether they play a role before or after mating. (Dobzhansky 1951; Coyne and Orr 1989, 1997; Rundle and Schluter 1998; Coyne and Orr 2004; Servedio 2004). Using representatives of the entire African haplochromine cichlid fish radiation, I measured the rate at which pre- and postmating incompatibilities accumulate between species with variable levels of genetic differentiation (**Chapter 4**). I measured premating isolation as the proportion of females not spawning with heterospecific males and postmating isolation using four components of hybrid viability measured at different ontogenetic stages from zygotes through to adulthood (controlling for pre- and postmating isolation in homospecific parental crosses). Genetic distance between species was translated into absolute evolutionary time using three different molecular clocks: one internally calibrated, linear clock, based on the biogeography of Lake Malawi (Sturmbauer et al 2001), one non-linear clock, calibrated to the cichlid fossil record plus recent geological events, and another non-linear clock calibrated to the breakup of Gondwanaland plus recent geological events (Genner et al 2007). In this chapter, I will base all my time estimates on the Gondwana clock because the timescales of cichlid evolution derived from fossil-dated phylogenies of other bony fishes most closely matched those suggested by Gondwanan fragmentation calibrations (Genner et al 2007). However, I will also report the estimates from the fossil clock although it has been shown to lead to an underestimation of divergence times, to be conservative with regard to predicting the window of opportunity for successful interspecific hybridization, which is one of the main aims of my thesis.

I found that compatibility was lost two to three times faster in traits affecting postmating isolation than in traits affecting premating isolation. Even though initially premating isolation was considerably high between closely related species, it then only increased by 4/2 % per million years (MY) divergence time (fossil record/Gondwana breakup calibration). Conversely, postmating isolation started off low with hybrids being similarly or even better viable than the homospecific control crosses but viability then dropped in a clock-like manner by 13/6 % per MY. Interestingly, the effects of divergence time on hybrid viability were distinctly ontogenetic stage-specific. While some stages were strongly affected by genetic incompatibilities that increased in a clock-like manner with the genetic distance

between species (i.e. fertilization rate and post-hatching larval survival), others were completely independent of the genetic distance between parents (hatching rate and juvenile survival). Complete inviability only occurred after 8.5/18.4 MY

We conclude that the rate at which intrinsic incompatibilities arise in the cichlid haplochromine radiation is by orders of magnitude lower than rates of speciation within individual lake radiations. Instead, these results suggest that intrinsic isolation mechanisms have little to do with the diversification of cichlid fish while other, extrinsic forces, such as divergent ecological and sexual selection, are the main drivers of cichlid fish diversification (Kornfield and Smith 2000; Seehausen 2000; Streelman and Danley 2003; Kocher 2004; Stelkens and Seehausen submitted). This notion is further supported by the findings from **Chapter 6**, where I show that genetic distance between species was not related to the strength of reproductive isolation, whereas ecomorphological distance, indicating divergent ecological adaptation, was a good predictor of behavioural reproductive isolation between species in a recent radiation.

I conclude that the maintenance of the Great African Lakes cichlid fish diversity largely depends on extrinsic mechanisms of genetic isolation. Because these can be easily reversible when the shape and magnitude of selection coefficients change, the cichlid species diversity is highly vulnerable because reproductive isolation can break down under changes in the environment and habitat disturbance (Seehausen et al. 1997).

Investigating the rates at which reproductive compatibility decays in cichlid fish is of particular relevance because cichlid evolution seems to be characterized by frequent hybridization (Seehausen et al. 1997) and potentially hybrid speciation (Salzburger et al. 2002; Schliewen and Klee 2004; Schelly et al. 2006; Day et al. 2007). In fact, hybridization between distantly related lineages has been suggested to have contributed to the generation of entire adaptive radiations of cichlids (Seehausen et al. 2003; Seehausen 2004; Joyce et al. 2005). **In Chapter 5** (see below), I describe a new, extensive cichlid radiation from Lake Mweru (Zambia/DRC) for which such a scenario seems to apply. In order to test the feasibility of this scenario for the diversification of this group, additionally to providing molecular genetic evidence, it is crucial to determine experimentally the amount of time that may elapse before hybrid viability and fertility cease.

The results from **Chapter 4** confirm that the proposed hybridization events between the potential ancestors of the different radiations (Seehausen et al. 2003; Joyce et al. 2005, **Chapter 5**) could have indeed resulted in viable offspring despite the long time period of independent evolution between them, supporting the feasibility of the hypothesized hybrid swarm origin of these species flocks.

It must be noted here that no study to date, including mine, investigated the ecological competitiveness of cichlid fish hybrids with their parental species. Besides intrinsic incompatibilities causing low hybrid fitness, postmating isolation can also result from extrinsic ecological or sexual selection against hybrids when these suffer from an ecological or mating disadvantage (Hatfield and Schluter 1999; Vamosi and Schluter 1999; Naisbit et al. 2001; van der Sluijs et al. 2008). In future experiments, it would hence be important to test the exogenous fitness of hybrids of varying genetic crossing distance, ideally in different genotype-environment combinations (i.e. testing hybrid fitness in both parental habitats, intermediate habitats, novel habitats), to better evaluate if hybridization could have indeed contributed to the adaptive diversity seen in haplochromine cichlids.

Hybridization facilitates adaptive radiation
The cichlid fish of African lakes and rivers are one of the best model systems for adaptive radiation research (Kocher 2004; Seehausen 2006). Their extraordinarily high rates of speciation and morphological evolution, and the large numbers of closely related, sympatric species are an enduring puzzle to evolutionary biologists. Some of the unresolved questions

are: Which role does ecological opportunity plays in determining the likelihood for radiation and how important are extrinsic, environmental mechanisms compared to intrinsic, organismal factors in the generation and maintenance of diversification?

In **Chapter 5** I describe a large diversity of haplochromine species in Lake Mweru (Zambia/DRC) representing a major radiation that had gone completely unnoticed to date. On a field trip in 2005, we identified more than 40 phenotypically distinct taxa, varying in, size, body shape, jaw morphology, male breeding colouration, and habitat use. Several of the taxa resembled well known lineages from outside the area. Others represented completely new phenotypes not known from anywhere else (among these a large number of diverse species closely related to the widespread but usually species-poor genus *Pseudocrenilabrus*).

Constructing a sample-rich mitochondrial genealogy of Lake Mweru's species diversity revealed that at least eight phylogenetically distinct lineages of haplochromine cichlids must have independently colonized Lake Mweru, which apparently resulted in four different sub-radiations in the genera *Sargochromis*, "large tooth" *Serranochromis*, "small tooth" *Serranochromis*, and *Pseudocrenilabrus*. Quantitative morphological analysis suggests that each of these new radiations contains minima of between five and 15 phenotypically distinct taxa. Strikingly, we found traces for hybridization in all four radiations, witnessed by mitochondrial haplotype capture between lineages, involving 1.7/2.9 MY (fossil record/Gondwana breakup calibration) divergent lineages of *Pseudocrenilabrus*, and three different 3.3-4.7/6.3-9.5 MY divergent lineages of serranochromine cichlids. Hybridization must have been ancient, probably before the species radiations started, as implicated by nuclear genomic monophyly of the Mweru serranochromine radiation despite very deep polyphyly of the constituent mitochondrial lineages. The subsequent diversification was then probably a response to selection in a heterogeneous lake habitat with its many opportunities for ecological diversification. A similar situation, revealing ancient origins of genetic diversity predating the age estimates of lake formation has been suggested for the cichlid faunas of Lake Victoria (Seehausen et al. 2003; Stager and Johnson 2007) and Lake Makgadikgadi (Joyce et al. 2005).

The experimental work I present in **Chapter 4**, on the rates of accumulation of genetic incompatibilities among African haplochromine cichlids, suggests that fully viable F1 generation hybrids are regularly obtained from species crosses with up to 4/8 MY time of divergent evolution, and that viability is completely lost only after 8.5/18.4 MY (Stelkens et al. submitted). The divergence times between the hybridizing ancestors of the Lake Mweru radiations fall within the scope of this experimentally defined window of opportunity, confirming the plausibility of the *hybrid swarm origin hypothesis* which states that genetically diverse hybrid swarms are more prone to radiate than populations descending from a single ancestor (Seehausen 2004). However, a subsequent study, continuing with the hybrids I bred, finds that viability was on average 40% lower in the F2 hybrid generation across all genetic distances measured (C. Schmid, Stelkens, Seehausen manuscript) indicating that hybrid fitness should be measured across several generations if we want to obtain a realistic estimate of the intrinsic evolutionary potential of hybrid populations.

Dating the onset of each sub-radiation using three different molecular clocks indicates that all four radiations must have started at a similar point in time between 0.18/0.94 MY ago. Analysis of morphological disparity shows that the four sub-radiations are complementary to each other in morphospace, with each clade occupying a distinct area. This could result from competition for resources constraining the direction and volume of phenotypic evolution within radiations and additionally perhaps other co-evolutionary mechanisms, such as predator-prey interactions acting during their diversification. The *Serranochromis* sub-radiations feature exclusively predatory species (medium sized to large pursuit and ambush hunters), the *Sargochromis* sub-radiation contains mainly insectivores and molluscivores, and the *Pseudocrenilabrus* radiation contains only small-bodied planktivores, algae scrapers and

other small particle feeders. Together, Lake Mweru's large phenotypic diversity resembles that of other Great African Lakes and harbours ecologically relevant shape variation comparable in magnitude to the classic examples of cichlid adaptive radiations making Lake Mweru yet another hotspot of haplochromine cichlid diversity.

In summary, our data suggest that the radiations of Lake Mweru are recent and that speciation is likely a result of hybridization between divergent lineages and adaptation to the highly heterogeneous lake habitat. These data are the strongest yet available evidence that ancient hybridization between distant lineages is associated with rapid diversification of cichlid species, and that competition between major lineages causes co-evolutionary patterns in the ecomorphological diversification of each sub-radiation.

Hybridization and ecological speciation

An unresolved question in the generation of adaptive radiations is how premating isolation is maintained between the incipient species that often occur in close geographic proximity in the absence of physical impediments to gene flow. Because ecological adaptation can cause rapid changes in the mating system, selection-driven evolution of mating preferences is expected to dominate over other mechanisms during adaptive radiations, when multiple available niches are colonized in short succession by the same ancestral population (Schluter 2001).

In **Chapter 6**, I used three sympatric putative species of the *Pseudocrenilabrus* radiation of Lake Mweru described in **Chapter 5**, to test 1) if these eco-morphologically divergent populations are indeed different species, and 2) to test the prediction of ecological speciation that the strength of premating isolation among species is predicted by phenotypic rather than genetic distance (McPeek and Wellborn 1998). Despite being closely related, the three putative species differ in male nuptial colouration, ecomorphology and habitat preferences, indicating different ecological adaptations. To compare the effects of strong ecological and phenotypic, but weak genetic differentiation of sympatric putative species with the effects of strong genetic, but weak ecological and phenotypic divergence between geographically isolated populations, I also tested for assortative mating between a member of the radiation and an unrelated population of *Pseudocrenilabrus* from the allopatric Lake Bangweulu, which represent the same generalist riverine ecotype presumably ancestral to the Lake Mweru *Pseudocrenilabrus* radiation.

I found significant assortative mating among the three sympatric putative species, but random mating between the allopatric populations. Phenotypic distance between species, but not their genetic distance, was a good predictor of the strength of premating isolation indicating that speciation in the young adaptive radiation of *Pseudocrenilabrus* results from divergent adaptation rather than from evolution in geographical isolation. This result supports the role of divergent natural and sexual selection driving ecological speciation (Schluter 1996, 2000, 2001; Rundle and Nosil 2005). It is possible that premating incompatibility in this system arose as a byproduct of divergent ecological selection on traits affecting mate choice via pleiotropy (Kilias et al. 1980; Dodd 1989; Vines and Schluter 2006). Alternatively, low 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, including divergent ecologically based sexual selection (Maan et al. 2006; Seehausen et al. 2008).

The results from **Chapter 6**, together with an increasing number of theoretical (Gavrilets 2003) and experimental studies (Rundle et al. 1998; Mooers et al. 1999; Rundle 2003), challenge speciation models that assume drift to be the most common cause of speciation (Mayr 1963; Turelli et al. 2001). To better understand the importance of drift effects versus selection effects on driving speciation in cichlids, it would be important to test for premating isolation in sympatric versus allopatric species pairs, an approach which has not yet been fully implicated in cichlid fish and deserves the attention of further research.

Another interesting notion of **Chapter 6** is 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 and may hence readily hybridize when coming into secondary contact, e.g. upon river capture. This result is in agreement with the conclusions of **Chapter 4**, that premating incompatibilities accumulate slowly in allopatry (2-9% per my divergence). It is also in agreement with the findings of **Chapter 5**, where I suggested that two allopatric *Pseudocrenilabrus* populations, one from Lake Mweru and one from Lake Bangweulu, hybridized in spite of the long history of independent evolution between them. The complete absence of premating isolation between these divergent lineages supports the scenario of ancient hybridization followed by adaptive radiation suggested in **Chapter 5**.

Hybridization generates phenotypic novelty

In **Chapter 4**, I determined the window of opportunity for evolution through hybridization by measuring the intrinsic fitness of hybrids with increasing genetic distance between the parental species. However, this does not fully resolve the question if hybrid populations are ecologically competitive to establish themselves in nature. Usually, hybrids are intermediate in appearance between the parental lines, which will often lead to a fitness disadvantage for hybrid genotypes in both parental habitats as they will tend to be less well adapted to the prevailing ecological conditions and will fall into the valley between two adaptive peaks. Yet, sometimes hybrids express phenotype values that lie outside of the parental phenotypic range. The occurrence of such extreme offspring phenotypes is referred to as transgressive segregation (Slatkin and Lande 1994; Rieseberg et al. 1999). Transgressive segregation has been shown to be a common outcome of interspecific hybrid crosses (59 % of 579 traits in plant hybrids and 31% of the 650 traits in animal hybrids were transgressive in Rieseberg (1999)).

Theoretically, transgressive traits can provide hybrid genotypes with novel adaptive potential that is not shared by either parental population. Populations composed of many such hybrid genotypes may then diverge from the parental species through the same mechanisms that play a role in classical ecological speciation (Seehausen 2004). Evidence for beneficial effects of transgressive morphological and ecophysiological trait values when colonizing previously underutilized peaks on a fitness landscape comes from extensive studies on *Helianthus* sunflowers, that conclusively demonstrate how transgression in key ecological traits can allow hybrids to invade new ecological niches (Schwarzbach et al. 2001; Lexer et al. 2003b; Rieseberg et al. 2003a; Gross et al. 2004).

In **Chapter 2** and **3**, I investigated the previously untested prediction that with increasing genetic distance between the parental species, transgressive segregation becomes more frequent in interspecific hybrid offspring. This prediction is based on the finding of independent quantitative genetic studies that transgression is usually caused by complementary gene action or epistasis (e.g. DeVicente and Tanksley 1993; Rieseberg et al. 2003b). Complementary gene action and epistasis become more frequent the more time has elapsed since divergent evolution began because an increasing number of quantitative trait loci become fixed for different alleles in the parental species, hence providing more opportunity for transgressive segregation the larger the genetic distance between species.

In **Chapter 2**, I analyzed published phenotypic data on plant and animal hybrids and calculated genetic distances between the hybridizing species pairs from published molecular sequence data. I then applied independent contrasts to calculate phylogenetically controlled regressions testing the effect of genetic distance on transgression frequency. In eudicot plants, I found a significantly positive relationship with genetic distance explaining 43% of the variance in transgression frequency. A total of 36% traits were transgressive.

Opposite to the signal in plants, the animal data revealed an unpredicted, but significantly negative correlation between transgression frequency and genetic distance. This result may be explained by the effects of phenotypic differentiation. Transgression is predicted to decrease with increasing phenotypic differentiation between the parental species. This is because divergent directional selection tends to purge the antagonistic allelic effects within QTLs and within species required for complementary gene action. Stabilizing selection on the other hand allows for the maintenance of alleles of opposite signs within species and results more often in complementary and epistatic gene effects. Hence, the effects of phenotypic differentiation can override the effects of genetic distance on transgressive segregation. Since there was a significant, positive relationship between genetic distance and phenotypic differentiation in the animal data set, it is possible that the expected positive effect of time since speciation was masked by the negative effects of phenotypic differentiation. In the plant dataset, the predicted effect of time since speciation on transgressive segregation was unconfounded by the potentially conflicting effects of phenotypic differentiation between species because the latter was unrelated to genetic distance.

Although the functional relevance and fitness values of the transgressive traits detected in this analysis are mostly unknown, under some ecological circumstances (especially when habitats become newly colonized or thoroughly altered) the increased working surface for selection generated by transgressive segregation may be beneficial to the establishment of hybrid populations. This may, under such circumstances, compensate for an average fitness loss through genetic incompatibilities (Hatfield and Schluter 1999; Via 2002).

In **Chapter 3**, I tested the same prediction again but used an experimental approach measuring the amount of transgression occurring in F1 and F2 generation hybrids of African haplochromine cichlid fish species. I crossed species pairs with gradually increasing genetic distances and varying phenotypic similarity. Transgression in overall body shape was quantified from photographs using landmark-based geometric morphometric methods and a thin-plate spline procedure. Mitochondrial D-loop sequences from GenBank were used to determine genetic distance between species.

I found that genetic distance explained 52% and 78% of transgression in F1 and F2 hybrids, respectively. This confirmed experimentally the finding of **Chapter 2** that transgression was extremely common and that the frequency of transgression linearly increased with genetic distance, at least in the F2 hybrid generation. In F1 hybrids, a u-shaped relationship between transgression and genetic distance was observed, perhaps suggesting that both heterosis and non-additive genetic effects caused the expression of extreme trait values. Phenotypic distance between the parental species did not predict transgression in either F1 or F2 hybrids in this experiment.

Chapter 2 and **3** demonstrate that both the time since speciation and phenotypic differentiation have to be taken into account to predict the frequency of phenotypic novelty emerging from interspecific hybridization. This has important implications for the potential interaction of hybridization and adaptive evolution, as hybridization can generate new genotypes with adaptive potential that did not reside in either parental population, potentially enhancing a population's responsiveness to selection.

The observed positive relationship between genetic distance and transgression is particularly interesting for those species groups, where hybridization between largely divergent lineages has been suggested to have taken place at the onset of their adaptive radiations (reviewed in Seehausen 2004). The findings of **Chapter 2** and **3** demonstrate the commonness with which transgression occurs in hybrids between divergent genomes, helping perhaps to understand the rapid rates of phenotypic evolution observed in some of the most recent adaptive radiations (e.g. the rapid adaptive radiations of Lake Mweru described in

Chapter 6). The potential of transgressive segregation to increase the working surface for selection, can provide the phenotypic diversification of a species group with new momentum without the long waiting time to new mutations and allow hybrid populations to undergo niche shifts (Lexer et al. 2003a; Nolte et al. 2005).
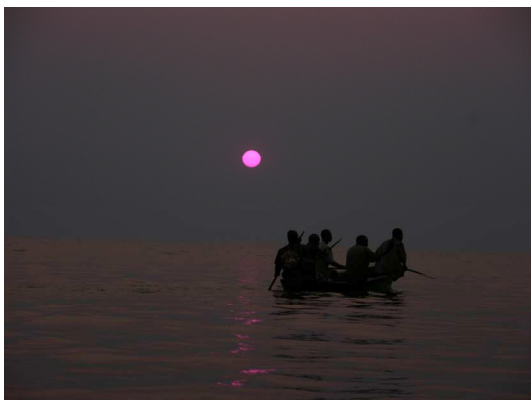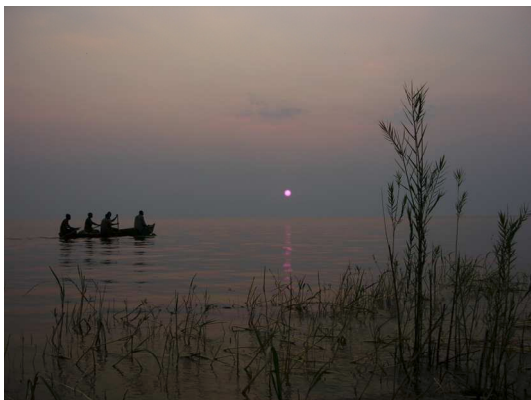
Future work could compare transgression frequencies in hybrids from phylogenetically controlled crosses between closely and distantly related species with both similar and divergent phenotypes to disentangle the effects of phenotypic and genetic distance on the amount of transgression in interspecific hybrids. If transgressive segregation was indeed an important contributor to the volume and extent of phenotypic diversification during adaptive radiations (Albertson et al. 2003; Seehausen 2004; Albertson and Kocher 2005), it would be interesting to quantify and compare the amount of transgression occurring between radiating (species-rich) and non-radiating species groups. Variation in the genetic architecture may cause varying degrees of complementary gene action in hybrids, which might help to explain why some lineages have massively radiated while others remained species-poor.

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<div align="center">Thank you!</div>

<div align="center">Rike</div>

Curriculum Vitae

Rike Bahati Stelkens

Address	Department of Ecology and Evolution, Biophore, University of Lausanne, 1015 Lausanne, Switzerland
	phone: +41 21 692 4249
	mobile: +41 76 339 7276
	fax: +41 21 692 4165
	email: rike.stelkens@unil.ch
	home: http://www.unil.ch/dee/page60981_fr.html
Private	Rue de la Dîme 19, 2000 Neuchâtel, Switzerland
Date of Birth	13 July 1978 (in Siegen, Germany)
Nationality	German
At Present	PostDoc (première assistante) in the lab of Prof. Dr. Claus Wedekind, Department of Ecology and Evolution, Biophore, University of Lausanne, Switzerland

Employment and Education

Nov 2008 - present	PostDoc (première assistante) in the lab of Prof. Dr. Claus Wedekind, Department of Ecology and Evolution, Biophore, University of Lausanne, Switzerland
Jan 2005 - March 2008	Doctoral Thesis work at the Department of Aquatic Ecology and Macroevolution, Institute of Zoology, University of Bern and the Department of Fish Ecology and Evolution, EAWAG, Switzerland
	Topic: *The role of hybridization in adaptive evolution*
	Supervisor: Prof. Dr. Ole Seehausen
	Examination committee: Prof. Dr. Michael Arnold, Prof. Dr. Laurent Excoffier
Feb 2005	Master's Degree, Examinations in Ecology (Grade: 1.3)* , Zoology (Grade: 1.0), Plant Pathology (Grade: 1.3)
Dez 2003 - Dez 2004	Master's Thesis under the supervision of Dr. Thomas Hoffmeister, University of Kiel, Germany. Lab work was performed at the University of Hull, UK, Department of Biological Science, in the Evolutionary Ecology Group of Dr. Ole Seehausen
	Topic: *Testing for disruptive sexual selection on male nuptial colouration by female mate choice in a Lake Victoria cichlid*
1998-2004	Study of Biology/Zoology at the Christian-Albrecht-University of Kiel, Germany (Oct. 2000: "Vordiplom" Examinations, Grade: 1.0)
1997-1998	"Au pair" and French language studies in Vevey, Switzerland
1988-1997	High School Christliches Jugenddorf Gymnasium Versmold, Germany
	Degree: Allgemeine Hochschulreife (Grade 1.4)
1984-1988	Primary School Bockhorst, Germany

* All grades according to the German marking system using grades from 1.0 (best) to 6.0 (worst).

Scientific and Work Experience

Sept–Oct 2005	Field work in the Republic of Zambia, Africa, Sampling of African cichlid fish
May–Aug 2004	Field assistant for Dr. Gert Petersen (Department of Agriculture, CAU Kiel)
	Project: Measuring aphid infection rates on wheat fields in Northern Germany to design an early warning system for pesticide treatment
Apr-Oct 2002	Research assistant at the CABI Bioscience Switzerland Centre, Delémont, Switzerland
	Projects: Classical biological control of invasive insect pests and weeds, investigation of host-parasitoid interactions, sampling of potential control agents in the field, virulence and host detection experiments under quarantine conditions
Apr–Oct 2001	Research assistant in the work group of Dr. Thomas Hoffmeister (Department of Ecology,

	CAU Kiel) Project: Analysis of the coexistence-aggregation-model and Allee-effects in *Drosophila*, finding evolutionary explanations for local species richness Methods: Lab experiments on egg laying behaviour, analysis of various life history traits, morphometrics of wing shape
Oct 2000-Apr 2001	Research assistant in the work group of Dr. Gregor Kölsch (Department of Zoology, University of Kiel) Project: Physiology, morphology, behaviour and biology of the aquatic leaf beetle *Macroplea sp.* (Chrysomelidae) Methods: Sampling, rearing and experimental investigation of mating and feeding behaviour

Grants and Scholarships

Jan 2008	Participation Grant sponsored by the Equal Opportunity Agency of EAWAG for the Workshop: Career planning for scientists, Ecole Polythechnique Fédérale de Lausanne, Switzerland. (400 EUR)
July 2006	Travel Grant sponsored by EAWAG to the conference: Genetics of Speciation in Vancouver, Canada. (1500 EUR)
Dez 2003	Research and Travel Grant from the Studienstiftung des Deutschen Volkes (German Scholarship Foundation). (3000 EUR)
June 2001	Member of the Studienstiftung des Deutschen Volkes (German Scholarship Foundation). Financial award for undergraduate studies. (5000 EUR)

International Meetings and Conferences (with presentation)

Sept 2008	Workshop: Managing adaptive variation in conservation biology, La Fouly, Department of Ecology and Evolution, University of Lausanne, Switzerland
Jan 2008	Workshop: Career planning for scientists, Ecole Polythechnique Fédérale de Lausanne (EPFL), Switzerland
Oct 2007	Seminar series: Aquatic Ecology and Macroevolution, EAWAG, Switzerland, Talk title: *The effect of genetic distance on novel trait expression in interspecific hybrids*
Sept 2007	Bioinformatics workshop: Phylogeny and Evolution using Bioinformatics, EMBnet, University of Lausanne, Switzerland, Talk title: *Four new adaptive radiations of haplochromine cichlids in Lake Mweru, Zambia*
March 2007	Conference: British Ecological Society Annual Symposium, Speciation and Ecology Sheffield, UK
Oct 2006	Conference: Hybridization in Animals- Extent, Processes and Evolutionary Impact, Frankfurt, Germany, Talk title: *Disruptive sexual selection by female mate choice on male colouration in interspecific hybrids*
July 2006	Statistics course series, EAWAG, Switzerland: "Introduction to R"
July 2006	Conference: Genetics of Speciation, The American Genetics Association (AGA),Vancouver, Canada, Poster title: *Disruptive sexual selection in hybrids between two sympatric cichlid species*
January 2006	Seminar series: Aquatic Ecology and Macroevolution, EAWAG, Switzerland, Talk title: *The impact of genetic divergence on segregation variance*
Aug. 2005	Conference: 10th congress of the European Society for Evolutionary Biology in Krakow, Poland, Symposium: Natural hybridization and speciation, Poster title: *Disruptive sexual selection in hybrids between two sympatric cichlid species*
June 2005	Seminar series: Aquatic Ecology and Macroevolution, EAWAG, Switzerland, Talk title: *The role of hybridization in adaptive evolution*
July 2004	PhD Workshop: Evolution and Ecology of Mate Choice, German Zoological Society, Lighthouse of Westerhever, Germany, Talk title: *Red or blue males? The role of female mate choice for speciation in a Lake Victoria cichlid*
May 2002	Conference: International Symposium on Animal Physiology, Trends and Developments in Animal Physiology, Greifswald, Germany, Poster title: *The new challenge: When leaf beetles (Chrysomelidae) become aquatic*

Teaching Experience

2008	Teaching assistance for seminar "Scientific writing", University of Lausanne, Switzerland
2008	Teaching assistance for lecture "Introduction into Conservation Biology", University of Lausanne, Switzerland
2008	Co-supervision of a Master student, University of Bern, Switzerland,
2006-2008	Teaching assistance for 2nd year Bachelor lecture "Evolution and Ecology of Biodiversity", University of Bern, Switzerland
2006-2008	Teaching assistance for seminar "Evolutionary dynamics and speciation", Lecture title: *Genetic variation, genetic constraint and evolutionary diversification*, University of Bern, Switzerland
June 2007	Teaching assistant for advanced student practical "Evolutionary Ecology and Morphology of Swiss stickleback populations, University of Bern, Switzerland,
2003-2004	Teaching assistant for undergraduate courses in Chemistry, Population Genetics and Evolution, University of Hull, UK

Publications and Manuscripts

1. **Stelkens, R.B.,** Seehausen, O. Genetic distance between species predicts novel trait expression in their hybrids, *in press, Evolution*
2. **Stelkens, R. B**., Pierotti, M. E. R., Joyce, D. A., Smith, A. M., van der Sluijs, I. & Seehausen,O. (2008)) **363**, 2861-2870, Disruptive sexual selection on male nuptial coloration in an experimental hybrid population of cichlid fish. *Philosophical Transactions of the Royal Society*
3. van der Sluijs, I., Van Dooren, T. J. M., Hofker, K. D., van Alphen, J. J. M., **Stelkens, R. B.** & Seehausen, O. (2008)) **363**, 2871-2877, Female mating preference functions predict sexual selection against hybrids between sibling species of cichlid fish. *Philosophical Transactions of the Royal Society*
4. **Stelkens, R.B.,** Young, K.A., Seehausen, O., Temporal patterns in the accumulation of reproductive incompatibilities between species of African cichlid fish*, submitted to PLOS Biology*
5. **Stelkens, R.B.,** Seehausen, O., Phenotypic divergence but not genetic distance predicts assortative mating among species of a cichlid fish radiation*, submitted to the Journal of Evolutionary Biology*
6. **Stelkens, R.B**., Joyce, D.A., Katongo, C., Seehausen, O., A new African Great Lake cichlid radiation emerged from five distantly related lineages through genetic exchange and competition*, manuscript*
7. **Stelkens, R.B**., Seehausen, O., Phenotypic novelty in experimental hybrids of cichlid fish is predicted by the genetic distance between species, *manuscript*
8. Kuhlmann, U., Gariepy, T., **Stelkens, R.B.** (2002) Predation on *Lygus spp*., *Annual Report CABI Bioscience Switzerland Centre*

Service as Reviewer

Philosophical Transactions of the Royal Society
Behavioural Ecology and Sociobiology

Further Skills

Languages	German: native speaker English: fluently spoken and written French: spoken and written
Lab and computer skills	• Standard laboratory techniques for genetic analyses: DNA extraction, agarose gels, polymerase chain reaction, sequencing , microsatellite genotyping, paternity analysis, AFLP (amplified fragment length polymorphism), library screening • Phylogenetics software: CEQ, Proseq, ClustalW, MEGA, BioEdit, PAUP, MrBayes, Phylip, Treeview, Arlequin, Fstat • Compiling databases and statistical packages for analyzing data: EndNote, EXCEL,

     JMP, R, SigmaPlot, SPSS, Statistica
- Morphometrics (traditional and landmark based geometric methods): TPS, Morphologika
- Digital picture analysis: Photoshop, ImageJ, Noldus Observer Pro
- Large scale meta analysis using online data bases: Web of Science, Genbank
- Operating Systems: MS Windows, MacOS X
- Text- and Dataprocessing: MS Word, Excel

Diving Licence	DTSA (German diving licence), valid worldwide
Driver's Licence	EU Class 3

Erklärung

gemäss Art. 28 Abs. 2 RSL 05

Name/Vorname: Rike Stelkens

Matrikelnummer: 05-129-747

Studiengang: Doktorat Biologie

Bachelor ☐   Master ☐   Dissertation X

Titel der Arbeit: *The role of hybridization in adaptive evolution*

Leiter der Arbeit: Prof. Dr. Ole Seehausen

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