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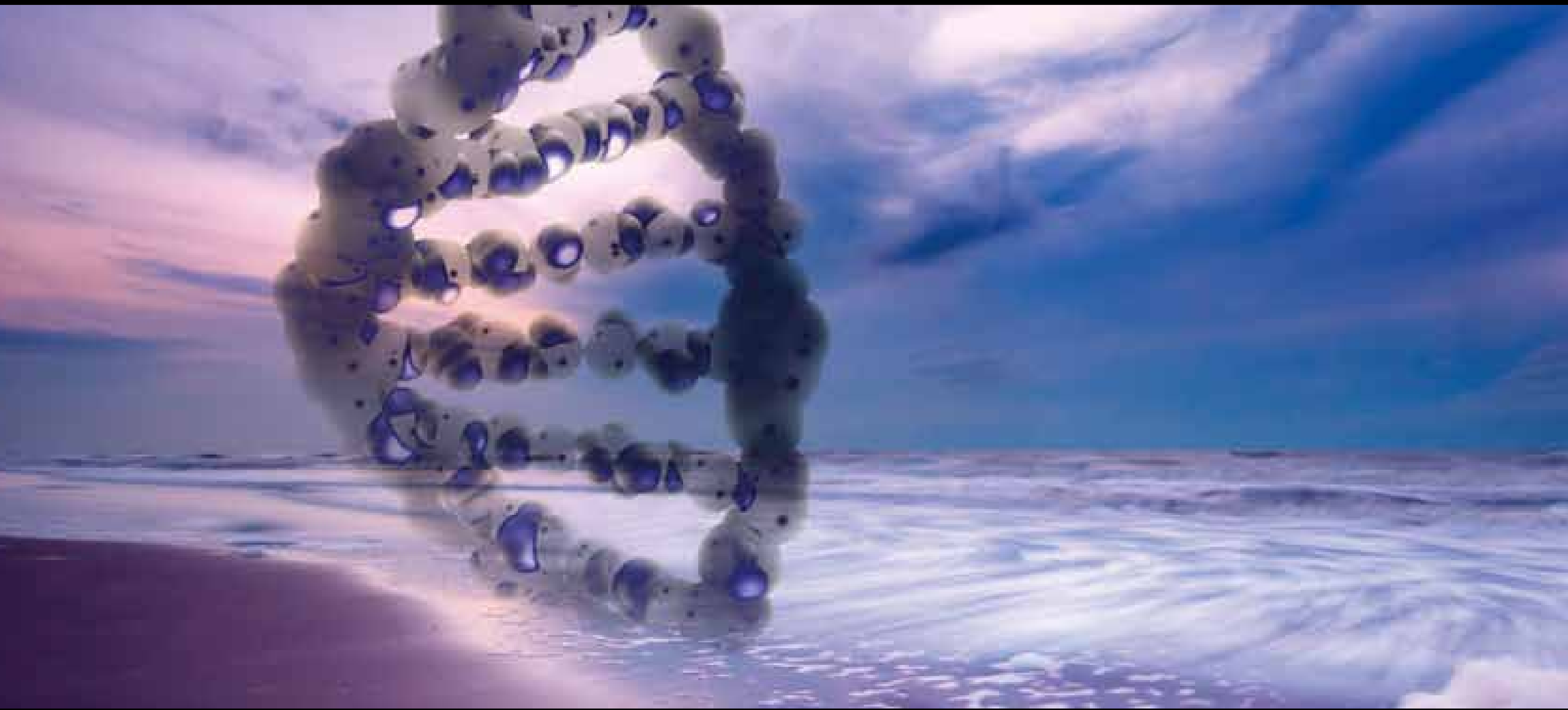

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Editorial Board

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Editorial

Cichlid Evolution: Lessons in Diversification

**Stephan Koblmüller,[1] R. Craig Albertson,[2] Martin J. Genner,[3]
Kristina M. Sefc,[1] and Tetsumi Takahashi[4]**

[1] *Department of Zoology, Karl-Franzens University, Universitätsplatz 2, 8010 Graz, Austria*
[2] *Department of Biology, University of Massachusetts, Amherst, MA 01002, USA*
[3] *School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK*
[4] *Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo, Kyoto 606-8502, Japan*

Correspondence should be addressed to Stephan Koblmüller, stephan.koblmueller@uni-graz.at

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With an estimated 3000 species, distributed from Central and South America, across Africa to Madagascar, the Middle East, and southern India, cichlid fishes (Cichlidae) represent the most species-rich family of vertebrates. In total they account for about 10% of extant teleost diversity. Throughout their distributional range, cichlids have repeatedly demonstrated their capacity for undergoing adaptive radiation, generating an outstanding variation of body shapes, color patterns, and behavior, and an enormous diversity of trophic and ecological specializations. This has made them an important model system for the field of evolutionary biology. With the completion of the first cichlid genome sequences, cichlid fishes are likely to receive even more attention in evolutionary research.

This special issue presents a collection of papers that tackle various aspects of cichlid biology essential for gaining a deeper understanding of factors and processes responsible for generating their diversity. Naturally, the selected topics and papers do not fully represent all fields of research that have contributed to our understanding of cichlid evolution. Nevertheless, they do represent rich and diverse knowledge which we are pleased to share with the readers of IJEB. Papers are summarized below in the order in which they appear in this special issue.

Lake Tanganyika is the oldest of the three East African Great Lakes and harbors the morphologically, behaviorally, ecologically, and genetically most diverse lacustrine cichlid species assemblage. In their review, "*The adaptive radiation of cichlid fish in Lake Tanganyika: a morphological perspective*," T. Takahashi and S. Koblmüller give a brief overview about recent phylogenetic findings and provide a synthesis of morphological studies that relate to the adaptive radiation of Lake Tanganyika's cichlids. They discuss many of the relevant studies that have contributed to our increasing knowledge of the evolutionary pathways and mechanisms generating this exceptional diversity within a short period of time. Furthermore, the authors highlight the potential importance of ontogenetic changes in morphology for establishing taxa in complex species-rich communities, a largely neglected issue in East Africa's species-rich lacustrine cichlid species assemblages.

Recent advances in morphometric methods provide great potential for detailed analyses of cichlid anatomy in an evolutionary context. In particular the establishment of geometric morphometric approaches has revolutionized the analysis of morphological data in cichlid evolutionary research. In their review, "*The utility of geometric morphometrics to elucidate pathways of cichlid fish evolution*," M. Kerschbaumer and C. Sturmbauer introduce geometric morphometric concepts and methods and also provide a summary of studies that employed this approach for analyzing and comparing cichlid morphologies. The authors promote the tremendous potential of this method for answering a variety of evolutionary questions involving complex shape changes in cichlid fish.

In the 1970s, Karel Liem demonstrated a functional decoupling between the oral and pharyngeal jaws in African cichlids. He postulated that this attribute had freed the oral jaws from the functional constraint of food processing, thereby allowing the oral jaws to evolve elaborate modifications for prey capture. Implicit to this assertion is a degree of independence of the oral jaws from the rest of the craniofacial skeleton. In their paper, "*Modularity of the oral jaws is linked to repeated changes in the craniofacial shape of African cichlids*," K. J. Parsons et al. tested the hypothesis that the oral jaw apparatus among East African cichlids represents a variational module. Their data offer strong support for the presence of such a module across all lakes as well as within each lake. This finding helps to explain why the direction of cichlid craniofacial radiations has been largely conserved across lineages.

The cichlid species of Lake Tanganyika are also the focus of another review, "*Mating and parental care in Lake Tanganyika's cichlids*," by K. M. Sefc. The paper starts with a comprehensive evaluation of field and genetic data on the mating and parenting behavior of mouthbrooding and substrate breeding cichlid species. The review then considers alternative reproductive tactics, including cooperative breeding, sneaking, and piracy. In the electronic format, this summary can easily be searched for information on particular species and genera. The review also addresses intraspecific variability in mating behavior, the evolution of cichlid mating systems and sexual dimorphism, and closes by emphasizing the difficulty of defining mating systems and sexual selection intensity at species level.

It is often assumed that the primary selective force driving divergence in sexually selected traits is direct female choice. However, there is another possibility: indirect selection operating through male-male competition. In "*Male-male competition as a force in evolutionary diversification: evidence in haplochromine cichlid fish*," P. D. Dijkstra and T. Groothuis review the role of male-male competition in cichlid divergence, focusing upon the haplochromine cichlids of Africa. They use examples to show how males with novel color patterns in a community may enjoy an advantage as they are subject to less aggression from established territorial males and speculate that this may contribute to speciation by allowing males with novel colors to establish territorial space in communities. They also discuss how aggression biases are not always symmetric between sympatric color morphs, and describe the evidence that the color of individuals and their aggression biases are genetically linked. The paper closes with intriguing proposals for future work. These include tests to establish how male-male competition and aggression biases are mediated by the availability of territorial space, and how selection on traits correlated with color, for example, physiological traits, may promote or constrain adaptive evolution.

The influence of male color pattern on female mate choice was examined in a research paper by O. Svensson, B. Egger and others, "*Segregation of species-specific male attractiveness in F2 hybrid Lake Malawi cichlid fish.*" In laboratory experiments with two sympatric, differently colored *Pseudotropheus* species, females were given a choice of hybrid males displaying a range of intermediate color patterns, and male mating success was determined by genetic analysis of broods. Although males differed in their attractiveness towards females, there was no correlation between male mating success and male coloration, and mate choice may have depended on nonvisual signals, segregating male preferences, or visual signals not quantified in the study. A quantitative genetic model estimated that only as few as two chromosomal regions may control species-specific attractiveness, which suggests that few genes with major effects can promote reproductive isolation in the radiation of Lake Malawi cichlids.

Environmental influences on color pattern evolution are the focus of a study on another Lake Malawi cichlid genus. In "*One fish, two fish, red fish, blue fish: geography, ecology, sympatry, and male coloration in the Lake Malawi cichlid genus Labeotropheus (Perciformes: Cichlidae),*" M. J. Pauers identified biases in the distribution of red coloration dependent on lake region, depth distribution, and the presence of a congeneric species. The latter observation connects to the two previous papers, as in areas of sympatry, color pattern differentiation may follow from intrasexual competition between similarly colored males or the requirement of mate recognition cues to maintain reproductive isolation. The correlations detected in this study underscore the importance of both sexual and natural selection for nuptial color pattern evolution. An interesting postscript to the discussion deals with the question of why fish employ red coloration rather than, for example, becoming ever bluer in their endeavor to signal efficiently to conspecifics lacking long-wavelength-sensitive photoreceptors, in a long-wavelength-deprived environment. M. J. Pauers suggests that the answer lies in within-pattern contrast and supports his claim with an instructive illustration of a protanopic's (human or fish) impression of *Labeotropheus* coloration.

The quantitative genetics underlying "animal personality" is expressed in the behavioral variation within and between individual animals and has hitherto been examined mainly in domesticated species. In "*Repeatability and heritability of behavioral types in a social cichlid*," N. Chervet and coauthors used the cooperatively breeding cichlid *Neolamprologus pulcher* to study long-term repeatability and heritability of behavioral types in a wild animal. Boldness, aggressiveness, and exploration propensity were repeatedly scored in 1779 individuals up to 1201 days apart and joined into a single measure of behavioral type. Despite considerable repeatability (which declined over time), low heritability suggested other, perhaps nongenetic, effects on the variability of behavioral type. Importantly, the paper identifies several methodological and conceptual issues relevant for studies on correlated behaviors, for instance, the need to determine repeatability and to assess the fitness effect of the studied behavior in a natural situation.

A familiar concept among evolutionary biologists studying cichlids is that philopatry of fish populations provides opportunities for local selective forces to drive evolution in allopatry. There is now a considerable amount of evidence for this among rock-dwelling lacustrine cichlids, but less so for species that occupy offshore habitats, or peripheral lagoons. In "*Low genetic and morphometric intraspecific divergence in peripheral Copadichromis populations (Perciformes: Cichlidae) in the Lake Malawi basin*," D. Anseeuw and coauthors tested for morphological and genetic divergence among populations of *Copadichromis* species in the Lake Malawi basin. They found that populations of both species showed slight genetic differences, but there was no clear evidence that the population in the peripheral Lake Malombe differed substantially in neutral genetic markers or body shape to the populations in the main lake. Perhaps one of the most striking aspects of the study is that the body sizes of both species sampled from Lake Malombe were considerably smaller than those in Lake Malawi. Given that Lake Malombe has been heavily fished for decades, the authors discuss the intriguing possibility that the observed contrast in body size is an evolutionary response to fisheries exploitation.

Recent evidence suggests that diversification and maintenance of divergence despite gene flow are much more common than previously assumed. In "*Community genetics reveal elevated levels of sympatric gene flow among morphologically similar but not among morphologically dissimilar species of Lake Victoria cichlid fish*," N. Konijnendijk et al. demonstrate this phenomenon by examining the genetic structure among populations of five species of Lake Victoria cichlids in each of four island communities. They show that allopatric conspecific populations of these five species are on average more strongly differentiated than sympatrically occurring heterospecific populations of morphologically similar species, consistent with the idea that maintenance or maybe even evolution of phenotypic divergence is possible in sympatry despite high levels of interspecific gene flow. Hence, large parts of the genome might be exchanged among species without dramatically affecting morphology. On the other hand, phenotypic similarity between conspecific populations from different islands can be maintained in the face of very little gene flow between these conspecific populations indicating that geographic isolation is apparently not the sole most important determinant of diversification in recently diverged (Lake Victoria) cichlids.

The classification of Discus (genus *Symphysodon*) populations into species has been a difficult and somewhat controversial subject. In their study "*A molecular perspective on systematics, taxonomy and classification of Amazonian Discus fishes of the genus Symphysodon*," M. V. Amado et al. investigated this issue using wide ranging sampling of 24 populations across the Amazon basin, comparing their genetic structure using a suite of 13 microsatellite loci. Their results showed the presence of four nuclear genetic groups, each largely corresponding to color morphs. Because of the broad scale of sampling, it was also possible to demonstrate that these genetic groups also differed in the average habitat use, a pattern consistent with evolutionary divergence in habitat preferences. Together the study clearly demonstrates the value of a broad-scale sampling and the use of multiple molecular markers to test hypotheses of species status and inform systematic revisions.

The exact age of origin of and diversification events within cichlid fishes is still a matter of discussion, and several alternative hypotheses have been put forward in recent years. In their strategy paper "*The monogenean parasite fauna of cichlids: a potential tool for host biogeography*," A. Pariselle et al. propose an alternative approach for tackling the origin and phylogeographic history of cichlid fishes. They suggest that phylogenetic and biogeographical analyses of dactylogyrid parasites might be helpful in elucidating the history of their cichlid hosts and discuss a preliminary morphology-based phylogenetic analysis on the genus level which fails to support one specific scenario. Hence, the authors emphasize that a much more comprehensive in-depth analysis including molecular data and parasites from various host families will be essential for providing enough resolution to contribute to solving this heavily discussed issue in cichlid evolutionary research.

Altogether, this is a diverse array of papers that demonstrate the breadth of research questions that are possible to address using the cichlid system. They also show how cichlid research can significantly contribute to development and understanding of evolutionary theory. We hope you enjoy reading the contributions.

Acknowledgment

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Stephan Koblmüller
R. Craig Albertson
Martin J. Genner
Kristina M. Sefc
Tetsumi Takahashi

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Review Article

The Adaptive Radiation of Cichlid Fish in Lake Tanganyika: A Morphological Perspective

Tetsumi Takahashi[1] and Stephan Koblmüller[2]

[1] *Laboratory of Animal Ecology, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo, Kyoto 606-8502, Japan*
[2] *Department of Zoology, University of Graz, Universitätsplatz 2, 8010 Graz, Austria*

Correspondence should be addressed to Tetsumi Takahashi, tetsumi@terra.zool.kyoto-u.ac.jp

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Lake Tanganyika is the oldest of the Great Ancient Lakes in the East Africa. This lake harbours about 250 species of cichlid fish, which are highly diverse in terms of morphology, behaviour, and ecology. Lake Tanganyika's cichlid diversity has evolved through explosive speciation and is treated as a textbook example of adaptive radiation, the rapid differentiation of a single ancestor into an array of species that differ in traits used to exploit their environments and resources. To elucidate the processes and mechanisms underlying the rapid speciation and adaptive radiation of Lake Tanganyika's cichlid species assemblage it is important to integrate evidence from several lines of research. Great efforts have been, are, and certainly will be taken to solve the mystery of how so many cichlid species evolved in so little time. In the present review, we summarize morphological studies that relate to the adaptive radiation of Lake Tanganyika's cichlids and highlight their importance for understanding the process of adaptive radiation.

1. Introduction

With an estimated number of about 3000 species, distributed from Central and South America, across Africa to Madagascar and southern India, cichlid fishes (Cichlidae) represent the most species-rich family of vertebrates, accounting for about 10% of today's teleost diversity [1, 2]. Throughout their distribution range cichlids have repeatedly demonstrated their capacity of forming adaptive radiations—explosive speciation with niche partitioning (reviewed in [3])—, generating an outstanding variation of body shapes, colour patterns and behaviour, and an enormous diversity of trophic and ecological specializations [4–6], which attracted numerous evolutionary biologists and established them as one of the prime model systems in evolutionary biology (e.g., [7–9]), but the greatest diversity of cichlid fishes is found in the East African Great Lakes [2].

Although endemic cichlid species assemblages are known from most East African lakes, the largest lakes Tanganyika, Malawi, and Victoria harbour a particularly rich fauna of cichlid fishes, with an estimated number of 250–800 species in each lake [1, 2]. Thus, the number of species seems to be correlated with lake size, congruent with the expectation that species diversity increases with habitat heterogeneity and with the opportunity for isolation by distances and allopatric diversification [9]. Because of high degrees of endemism (95–99%), these cichlid radiations most likely originated via intralacustrine speciation [1, 7, 10]. With an estimated age of 9–12 Myr [11], Lake Tanganyika is by far the oldest of these lakes, and thus harbours the morphologically, behaviourally, ecologically, and genetically most diverse species assemblage [1, 2, 12–15], although the number of species is the smallest. Currently 200 valid species are recognized [10] with several more awaiting scientific descriptions such that the total number of Lake Tanganyika's cichlid species has been estimated to 250 [1]. These 200 species have been classified into 12 [16] or alternatively 16 [17] tribes, largely supported by molecular data [10].

The specialized, diverse morphologies of these fish appear to be the result of adaptations in their respective niches [18], and thus, these fish are regarded as ideal model system for the study of adaptive radiation [4, 8, 9]. Schluter [19] defined adaptive radiation as "the differentiation of a single ancestor into an array of species that inhabit a variety of environments and that differ in traits used to exploit those environments," and employed four features as criteria to detect adaptive radiation: (1) common ancestry, (2) phenotype-environment correlation (empirical evidence of correlation between the diverse phenotypes of descendant species and their divergent environments), (3) trait utility (experimental or theoretical tests of performance or fitness of a trait in its corresponding environment), and (4) rapid speciation. The first and fourth criteria are the subject of phylogenetic analyses of the species assemblage. The second and third criteria are to find adaptive phenotypes that differ between species as a result of divergent natural selection. These adaptive phenotypes may have caused reproductive isolation between species as byproduct (ecological speciation, e.g., [20, 21]) or allowed co-occurrence of two or more closely related species at the same place and in the same time (resource partitioning, e.g., [22, 23]).

Within the Lake Tanganyika cichlid species flock species and species groups differ in many morphological traits. Numerous morphological studies on Lake Tanganyika cichlid fish have been published in the past. In this short review, we summarize the findings from these studies and highlight their importance for understanding the process of adaptive radiation.

2. A Quick Overview on Large-Scale Phylogenetic Patterns and the Age of the Species Flock

The first molecular phylogeny of Lake Tanganyika cichlids was published by Nishida in 1991 [32]. This allozyme-based phylogeny resolved the relationships of 20 species representing all Lake Tanganyika cichlid tribes and suggested that the Lake Tanganyika cichlids were polyphyletic. Subsequently, many molecular phylogenetic studies have been published, such that, in contrast to the situation in the much younger cichlid species flocks of Lakes Malawi and Victoria, we now have rather precise knowledge on the phylogenetic relationships within the Lake Tanganyika cichlid species flock and the relationship among African lacustrine and riverine cichlid faunas (Figure 1) (reviewed by [8–10]).

With the exception of *Tylochromis polylepis* and *Oreochromis tanganicae*, the Lake Tanganyika cichlids evolved from a common ancestor after the formation of the lake 9–12 Ma [24, 27, 33]. These fish are thought to have rapidly radiated within the lake, which fulfils the first and fourth criteria of adaptive radiation presented by Schluter [19] (common ancestry and rapid speciation). *Tylochromis polylepis* and *O. tanganicae* colonized the lake only recently, thus establishing themselves in an already mature adaptive radiation [25, 31]. Excluding these species, the Lake Tanganyika cichlid species flock comprises at least six major lineages [27]. Two of these lineages comprise many morphologically highly diverse species [34]. The substrate-brooding tribe Lamprologini consists of about 80 species endemic to the lake, plus eight species that colonized the Congo River [35] and one species that colonized the Malagarazi River [36] after the intralacustrine radiation of this tribe [37, 38]. The mouth-brooding C-lineage (sensu [24]) includes about 100 endemic species assigned to six [16] or ten [17] tribes (species numbers, phylogenetic relationships, and biological characteristics of tribes are reviewed in [10]). Whereas the monophyly of each tribe is well supported, the phylogenetic relationships among the tribes are still largely unresolved, indicating rapid diversification and adaptation to particular ecological niches at the onset of the Lake Tanganyika radiation. The Tropheini, one of the endemic mouth-brooding tribes, were shown to be nested within the haplochromines, the most species-rich lineage that also includes the species flocks of the remaining East African Great Lakes and the majority of the northern, eastern, and southern African riverine cichlid species but originated in the course of the primary Tanganyika radiation [29, 30]. The simultaneous radiation of the Lamprologini and the C-lineage was probably triggered by the onset of deep-water conditions in Lake Tanganyika about 5–6 Ma (primary lacustrine radiation) [27, 30, 38]. Alternative hypotheses [39, 40] suggest a two- to fivefold older age for the Lake Tanganyika radiation which would considerably predate the establishment of a real lacustrine habitat [11, 41, 42] and imply that the onset of the radiation has happened in a riverine environment, a habitat generally considered as not suitable to host a radiation [43]. The biological characteristics of the Lake Tanganyika cichlid species assemblage with a clear resource partitioning between most tribes and the relative age of the East African cichlid species flocks strongly argue for the Lake Tanganyika radiation to have happened in a single deep-water lake [10, 30].

Unlike for the younger Lakes Malawi and Victoria molecular phylogenies of Lake Tanganyika cichlids are typically well resolved, with most species, genera, and tribes being resolved as monophyletic, indicating that lineage sorting has been largely completed. Thus, it has been assumed that these molecular phylogenies (typically based on mitochondrial genes) do closely approach the true species trees although the placement of some taxa was inconsistent with taxonomy [44–46]. Recent evidence from complementary analyses of nuclear DNA, however, clearly demonstrated that, despite being well resolved, the mitochondrial phylogenies do not necessarily reflect the true phylogenetic relationships but might be severely misleading due to ancient incomplete lineage sorting, ancient (and recent) introgression, and even hybrid speciation [26, 38, 47–53]. Nevertheless, mitochondrial phylogenies have been and are still used as proxies of species trees in comparative approaches to study the interaction and evolution of biological traits in a phylogenetic context (e.g., [54–57]), thus potentially introducing an error in inferred evolutionary patterns.

3. Adaptive Morphology

The evolutionary success of cichlids has been attributed to the interaction of extrinsic environmental factors and intrinsic species-specific traits. Some of these intrinsic traits might be naturally selected (e.g., the trophic morphology, body size, body shape, and visual pigments), whereas others are predominantly sexually selected (e.g., body colouration,

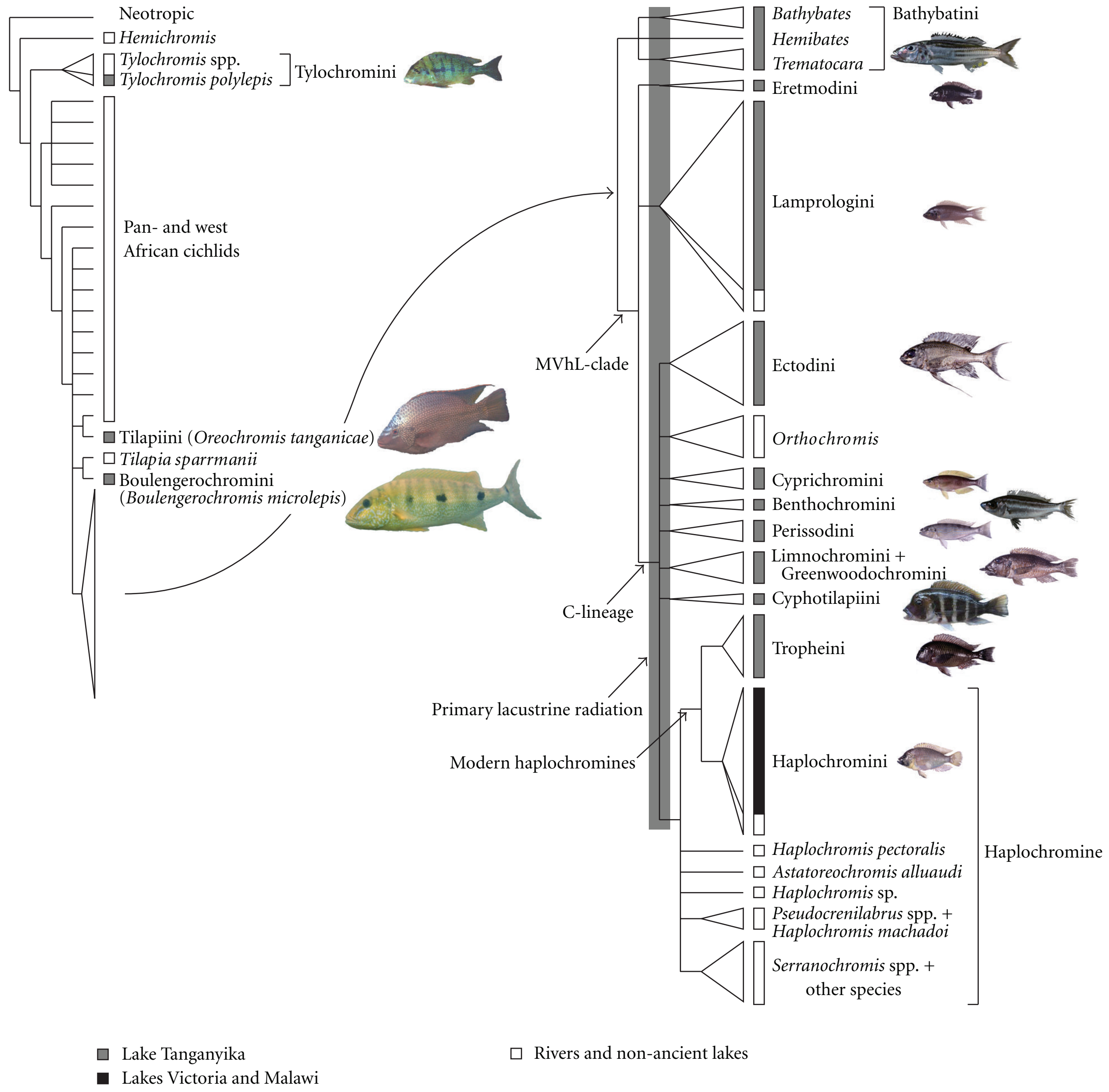

Figure 1: Schematic molecular phylogenies of the Lake Tanganyika cichlid assemblage based on combined evidence from several studies: [24, 25] for the phylogenetic tree on the left, that shows relationships among major African cichlid lineages, and [26–30] for the phylogenetic tree on the right, that shows a more detailed picture of the intertribal relationships of East African cichlids. Tribe names follow [17]. *Tylochromis polylepis* and *Oreochromis tanganicae* colonized the lake only recently and hence established themselves in an already mature adaptive radiation [25, 31]. In the phylogenetic tree on the right, lineages that underwent radiation are indicated by triangles, with their sizes corresponding to the number of species within the lineage (except for the non-Lake Tanganyika lineages). MVhL-clade was designated by [26], C-lineage by [24], and modern haplochromines by [29]. The Tropheini were shown to be nested within the haplochromine, the largest cichlid tribe that seeded the radiations of the other East African lakes and comprises the majority of North, East, and South African riverine cichlid species [29, 30].

smell, and courtship sounds), though distinctions between naturally and sexually selected traits might be not that clear (reviewed in [58]). Many authors have studied morphological traits in Lake Tanganyika cichlids in terms of adaptation. These studies may be classified into four major groups: studies of trophic morphology, body shape, body size, and nervous system.

3.1. Trophic Morphology. Cichlid fishes exhibit a functionally decoupled set of jaws, the oral jaws and the pharyngeal jaws (Figure 2). In particular the pharyngeal jaw is considered a key innovation, representing a key factor for the emergence of a diversity unparalleled among vertebrates [59–61]. The pharyngeal jaw apparatus of cichlid fish is a functionally integrated and highly specialized system and considered to

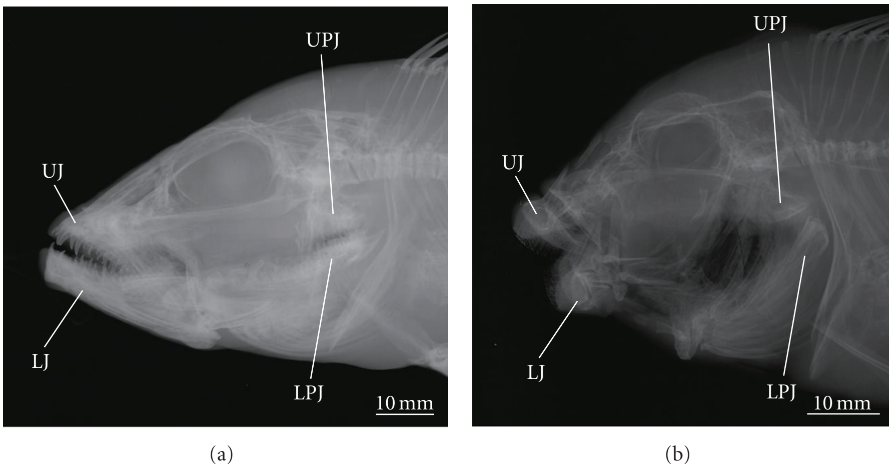

Figure 2: X-ray photograph of the head of (a) *Lamprologus lemairii* (piscivore) and (b) *Petrochromis trewavasae* (herbivore). LJ and UJ: lower and upper jaws, LPJ and UPJ: lower and upper pharyngeal jaws.

represent a major adaptive complex [59]. Decoupled from the oral jaws, the pharyngeal jaws are used for efficient crushing and processing of food items. Thus, the oral jaws are freed from their dual task of food collection and preparation. Due to this division of functions the development of numerous specializations of food collection and procession mechanisms became possible [59] and minor modifications in oral and pharyngeal jaw structure allow for the utilization of novel food resources within a few generations, such that unexploited ecological niches can be rapidly occupied [62, 63]. Thus, the specialized pharyngeal jaws in cichlid fish seem to be particularly important for their propensity to rapidly adapt to novel ecological niches, but other trophic morphological features have probably also played key roles for their rapid diversification. Likely, rapid differentiation in trophic specializations results in effective resource partitioning and thus drives the evolution of complex cichlid communities by ecological segregation (e.g., [64, 65]).

Several studies on Lake Tanganyika cichlids described correlations of the trophic morphology with food habits (oral jaws and teeth [65–69], pharyngeal jaws [59, 69, 70], preorbital region [15, 71], and intestine [72, 73]), suggesting resource-based divergent selection as an important diversifying force in Lake Tanganyika cichlids. In algae feeding species, for example, the shape of jaw teeth differs according to the resources they exploit. Thus, the species of the genus *Petrochromis* have tricuspid teeth in high density that make a brash-like structure to comb unicellular algae from filamentous algae on rocks, whereas species of *Tropheus* and some other genera have large bicuspid teeth in the most anterior row that allow to nip and tear off filamentous algae from rocks [66]. Tooth shape—both on oral and pharyngeal jaws—in cichlid fish was shown to respond quickly to selection and change even within the lifetime of a single individual [74, 75]. Recently, significant advances regarding the developmental pathways and genetic basis leading to different tooth shapes and numbers have been made [76–78]. Further work on this issue will certainly be important to fully understand how inter- and intraspecific differences in tooth shape and numbers emerge as a sometimes quick response to environmental/trophic constraints in species-rich adaptive radiations where species typically occupy a rather narrow ecological niche.

Besides the number and shape of teeth, the shape of the pharyngeal jaws and the oral jaws (or the preorbital region in general) are well known to strongly correlate with diet and were shown to respond quickly to natural selection when new habitats are colonized or when it comes to optimize feeding performance throughout ontogeny. Thus, piscivorous fish were shown to have longer oral jaws than insectivorous and herbivorous fishes [15], and likewise, slender and elongated pharyngeal jaws have been found to be highly correlated with piscivory [79, 80]. However, recent evidence on the Lake Tanganyika cichlid *Lepidiolamprologus elongatus* suggests that the mature piscivorous morphotype is refined by a relative widening of the caudal part of the lower pharyngeal jaw, which has been interpreted as prerequisite to the insertion of well-developed musculature and the construction of a powerful lever system which allows for processing large prey fish and relying on exclusive piscivory [70]. The length of the intestine typically varies in the order of piscivores < invertivores < herbivores, and hence diet has been shown to be a good predictor of intestine length at both intra- and inter-specific levels in Lake Tanganyika cichlid fish, indicating that plasticity in intestine length in response to diet is a further important mechanism for driving trophic shifts in adaptive radiations [73].

Correlations between trophic morphology and diet quality were recognized even when accounting for phylogeny in the shape of oral jaw teeth [81] and intestine length [73]. Though divergent natural selection on trophic morphologies leading to rapid morphological change has been shown to be of tremendous importance for the emergence of Lake Tanganyika's astounding cichlid diversity, stabilizing selection seemingly prevents further drastic changes in structures relevant for trophic specializations if species are already well adapted to a particular niche within a multispecies assemblage [82]. Balancing selection on trophic traits, on the other hand, may have acted to maintain intrapopulation polymorphism in Lake Tanganyika cichlids [83, 84]. The scale-eating *Perissodus microlepis*, for example, has morphological dimorphism in the mouth-opening direction to right and left [85]. This deflection of mouth has been shown to be heritable [86, 87], though environmental factors can influence the formation of this mouth laterality through development [88]. Several population models have demonstrated that this lateral dimorphism is maintained by negative frequency-dependent selection, resulting from interactions between predator and prey species (e.g., [89, 90]). An empirical study has suggested that disassortative mating between right-mouthed individuals and left-mouthed individuals stabilizes this intrapopulation dimorphism more strongly than only negative frequency-dependent selection acted [91].

In recent years, significant advances have been made towards the understanding of the genetic basis of differences in the cichlids' jaw morphologies [92–94]. Yet, further work on the genetic determination of jaw morphologies, the genetic basis of other diet-related traits (e.g., pharyngeal bone and intestine), and studies on the efficiency of particular trophic morphologies in feeding, processing, and digesting food items are required to gain further insights into the mechanisms underlying the rapid generation of a multitude of trophic types as adaptation to particular resources in Lake Tanganyika cichlid fishes.

The trophic specialization of the pharyngeal jaws may also lead to reproductive isolation between species via mate choice. Cichlid fish produce sound during male courtship display [95, 96], and this sound appears to be produced by stridulation of the pharyngeal jaws [97]. Courtship sounds have been shown to differ among closely related species of Lake Malawi cichlids [96, 98]. Thus, if females use male courtship sound as a cue for mate choice, inter-specific differences in sound may possibly cause assortative mating, and consequently reproductive isolation [95, 97]. The Lake Tanganyika cichlids are highly diverse in pharyngeal jaw morphology, and thus, mate choice based on courtship sounds might represent an additional mode of diversification or prevent heterospecific pairings between already differentiated closely related species, a hypothesis that calls for further detailed investigations.

3.2. Body Shape.

The influence of phylogeny on the evolution of body shape is small in the Lake Tanganyika cichlids, and it appears that body shape evolution is strongly affected by feeding habits [18]. Piscivorous fish, for example, typically have a much larger head and benthivorous fish tend to have a slender body [18]. Thus, body shape is not independent from trophic morphology [99]. The body shapes are generally associated with swimming modes in fish (e.g., [100–102]), suggesting that the divergent body shapes of the Lake Tanganyika cichlids also relate to other ecological factors, such as the efficiency of escaping from predators.

In some maternally mouth-brooding species, the size and shape of the head differ between sexes. This sexual dimorphism in head size and shape appears to be related to a larger buccal cavity in females [103, 104], suggesting that the evolution of body shape is partly associated with reproductive ecology. In the *Tropheus moorii* species complex, differences in the orientation of the mouth and the head profile have been observed among populations, despite a lack of obvious differences in food preferences, mating, and breeding behaviour [104, 105], indicating that these observed difference in body shape might be due to random genetic drift. Genetic drift can evolve phenotypes without adaptation, and perhaps it can take populations through adaptive valleys and into the domains of new adaptive peaks (peak shifts by drift, reviewed in [19]). Nevertheless, the Lake Tanganyika cichlid species have to establish themselves in a densely packed multispecies community. Consequently intraspecific morphological variation is typically rather low in the natural environment due to inter-specific competition and narrow ecological niches confining the morphospace occupied by a single species. If environmental conditions change, the phenotype might rapidly adapt to the new environment. This phenomenon has been recently shown by comparing morphologies of individuals of four wild populations of the *Tropheus moorii* species complex with their pond-raised F_1 offspring [106]. The extent of morphological change between wild and pond-raised F_1 fish was 2.4 times larger than the pairwise population differences, and all four populations exhibited the same overall trend in morphological change.

In the Lake Tanganyika cichlids, the diversity of body shape may at least partly be associated with adaptive radiation of the fish through resource partitioning, assortative mating, low hybrid fitness, and/or other mechanisms. However, nothing is known about the genetic basis of body shapes and also detailed studies on the efficiency of particular body shapes in feeding and swimming are lacking, thus opening a huge field of research for the future.

3.3. Body Size.

Body size is one of the simplest characters that show diversity between species. Body size evolution results from a balance between selection favouring large body size and selection favouring small body size (e.g., [107, 108]). While it is widely agreed that fecundity selection for females and sexual selection for males are the major evolutionary forces that favour larger body size in most sexual animals, counterbalancing selection favouring small body size is often masked by the good condition of the larger individuals and is therefore less obvious [108]. In the Lake Tanganyika cichlid fish, body size greatly varies from 42 to 650 mm in standard length (the smallest species is the shell-brooder *Neolamprologus multifasciatus*, and the largest species is the substrate brooder *Boulengerochromis microlepis* [34]). The effect of environmental constraints on body size has been studied in *Telmatochromis temporalis*. This species comprises dwarf and normal morphs, and the two morphs were shown to have evolved through divergent natural selection, in which the small body size of the dwarf morph was a result of adaptation to utilize empty gastropod-shells as shelters [109, 110]. The trait utility of body size has been examined in another shell-brooder, *Lamprologus callipterus*, that shows the most extreme male-biased sexual size dimorphism among animals (males > 12 times heavier than females [111]). Males of this species collect empty gastropod-shells to build up their nests. This behaviour is unique among the Lake Tanganyika cichlids. Experimental and theoretical tests suggested that the large male size of this species was determined by the ability to carry empty shells [112] and intersexual selection [113], and experimental tests suggested that the female size was limited by the ability to spawn eggs inside the shells [112].

Size-assortative mating has been reported in a wide range of animal taxa (e.g., planarians [114], snails [115], gammarus [116], insects [117–119], fishes [120, 121], toads [122], snakes [123], lizards [124], mammals [125], and birds [126]) and can cause reproductive isolation between species or morphs as byproducts of differences in body size [127–129]. In the Lake Tanganyika cichlid fish, divergent natural selection on body size might have been contributed to at least a part of explosive radiation of this species flock through assortative mating, and further, other various mechanisms such as low hybrid fitness and resource partitioning. Further work on intrinsic and extrinsic factors that affect the body size may give deep insights into the mechanisms underlying the adaptive radiation of these fish.

3.4. Nervous System. Some studies have described correlations between nervous system and ecology. Huber and colleagues [130] and Pollen and colleagues [131] demonstrated that the relative development of various brain structures relates to habitat, social behaviour, resources, and environment. Thus, in the tribe Ectodini for example, telencephalon, that appears to be involved in a variety of tasks, such as processing olfactory, visual and gustatory stimuli, and in learning, agonistic and courtship behaviours [130], was larger, and hypothalamus, that appears to have integrative functions relating to feeding, aggression, reproduction, and vision [130], was smaller in monogamous species compared to polygamous species. These studies suggest that selection or drift can act independently on different brain regions. Gonzalez-Voyer and colleagues [57] showed that female brain size correlated with brood care type and diet type and that male brain size correlated with diet type only, suggesting that more complex diet selection and larger burden on brood care may demand larger brain size. Sylvester and colleagues [132] showed that an alternative SNP in *irx1b* potentially causes differences in the relative size of the telencephalon versus the thalamus between rock-dwelling and sand-dwelling Lake Malawi cichlids. Although it is not at all obvious how an increase in the size of brain would give rise to functional differences (e.g., increased cognitive abilities) [133], the brain morphology may have played important roles in adaptive radiation of the Lake Tanganyika cichlids.

3.5. Other Morphologies. We reviewed a trophic morphology, body shape, body size, and nervous system, morphological traits that might be causally involved in the adaptive radiation of the Lake Tanganyika cichlids. However, these fish are highly diverse also in other morphological traits. The diversity of these other traits may be a result of adaptation to various environments, genetic drift, or phenotypic plasticity during development. Unfortunately, hardly anything is known about function and genetic basis of these traits. For example, the infraorbitals (a series of bones surrounding the lower half of the eye) were shown to vary considerably in shape among the Lake Tanganyika cichlids (Figure 3) [134]. However, it is still unknown what exactly caused the observed inter-specific differences in this morphological structure, though it has been argued that infraorbitals have the function to regulate the movement of jaws in relation to some other bones [15], and that the number and size of sensory pores on these bones may be associated with the noise sensitivity [135].

Some morphological characters differ at various taxonomic levels, reflecting difference in the relative time of morphological divergence. For example, the shape of infraorbitals tends to differ between tribes, suggesting that this morphology diverged during the initial radiation. The body shape and the shape of oral jaw teeth, on the other hand, tend to differ at lower taxonomic levels, such as between genera and between species, suggesting that the divergence of these morphologies reflect later evolutionary events.

4. Ontogenetic Changes in Morphology: A Largely Neglected Issue in Lake Tanganyika Cichlids

Studies on adaptation and natural selection typically focus on traits in adult organisms, but high mortality among juveniles indicates that—in addition to predation pressure—strong selection pressure, competition avoidance, and resource partitioning are presumably important already early on in life (e.g., [136]). Thus, individuals do not only have to compete for resources against heterospecific individuals but also against conspecifics. For species that comprise a number of cooccurring size-classes (many invertebrates, fish, amphibians, and reptiles) niche separation by body size differences poses a complex problem since the smallest size-classes of one species often overlap with the largest of another [137].

Since resource utilization abilities and predation risk are generally related to body size, many species undergo—sometimes dramatic—ontogenetic shifts in habitat use and/or food choice [138]. Thus, among fish, ontogenetic changes in resource use are nearly universal and size-related shifts in food choice have been documented in numerous species, typically with positive correlations between food size and body size (e.g., [139–145]). These ontogenetic shifts in resource use might vastly complicate species interactions with important consequences for community dynamics, in particular in multispecies communities [138]. If small and large species coexist, the most critical feature of this interaction is not how adults of these two species interact, but how the larger species is able to recruit through juvenile stages that are identical to the size ranges present in the smaller species. Interactions of this sort might form bottlenecks in recruitment to the species [146], and thus adaptive fine-tuning is particularly important in juvenile stages, especially

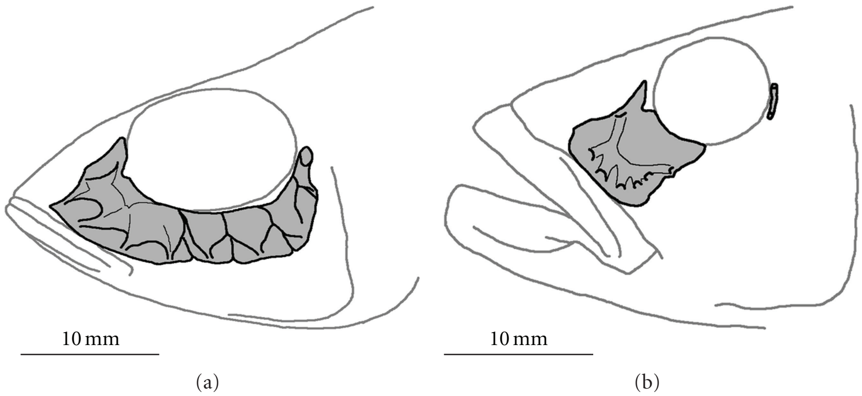

Figure 3: Infraorbitals of (a) *Trematocara unimaculatum* (Bathybatini) and (b) *Lepidiolamprologus profundicola* (Lamprologini). The infraorbitals vary in the number of bones, the degree of development of bones, and the numbers and size of sensory pores on the bones.

in regions/periods where/when resources are limited. Ontogenetic niche shifts aid in maximizing fitness by reducing competition with conspecifics via resource segregation [138], by minimizing predation risk through habitat shifts [147], and by maximizing growth through dietary shifts [148]. These ontogenetic shifts in resource use might be rather abrupt and are often correlated with discrete growth periods in the life history (e.g., [149, 150]). For many primarily piscivorous fishes the transition to piscivory is a crucial ontogenetic niche shift [151]. Typically, highly specialized piscivores are not particularly well adapted to feeding on zooplankton and benthic invertebrates, which are their predominant prey early in life [138, 151], and delayed shifts to piscivory can result in slow growth and increased mortality, in particular when competing for resources with specialist planktivorous species [138, 151, 152]. The switch to piscivory often initiates an increase in growth rate, translating into larger body size and greater survival—larger individuals are typically less vulnerable to predation and are better adapted to survive periods of starvation—throughout life for specialized piscivores [151, 152]. Transition dates might vary considerably among species (e.g., [151]). Several factors, for example, species-specific differences in the allometry of trophic structures, hatching time and size at hatching, have been proposed to at least partially explain these inter-specific differences in the timing of the switch to piscivory [151, 153]. Although there is a considerable amount of studies relating dietary shifts to ontogenetic changes of overall body shape in fishes (e.g., [139–141, 144, 145]), studies that directly relate ontogenetic changes in diet and growth patterns of the trophic apparatus are scarce (e.g., [142]). Whereas isometric growth of the lower pharyngeal jaw was reported for the Lake Tanganyika cichlid *Lamprologus ornatipinnis* [69], a species that predominantly feeds on invertebrates and thus does not experience a drastic shift in feeding habits throughout ontogeny, Hellig and colleagues [70] showed that an allometric change in ontogenetic lower pharyngeal jaw development of *Lepidiolamprologus elongatus*, a top predator in the shallow rocky habitat of Lake Tanganyika, coincides with the dietary shift to exclusive piscivory (Figure 4). This observation might indicate that distinct allometry is correlated with strong specialization, but it remains to be tested whether this is a general phenomenon in trophic specialists, what is the genetic basis of such morphological changes, and to what extent differential gene expression producing differences in morphology contributes to the astounding diversity of cichlid fishes in Lake Tanganyika.

5. Conclusions

Along with the Darwin's finches from the Galápagos Islands (e.g., [158]) and the Hawaiian silverswords (e.g., [159]) the East African cichlid species flocks represent well-established model systems for the study of adaptive radiation. Numerous morphological studies on the Lake Tanganyika cichlid species flock have greatly contributed to the ever-increasing knowledge on the evolutionary pathways and mechanisms generating tremendous diversity within a short period of time. Rapid changes in particular morphological traits allow for the rapid adaptation of cichlid fish to novel resources. Thus, such adaptive phenotypes would have played important roles during adaptive radiation. The Lake Tanganyika cichlids are highly diverse in their morphology, and many morphological traits appear to have been concerned with adaptive radiation. However, the knowledge about adaptive phenotypes is still poor. Clearly, more studies are required to reveal the mechanisms of adaptive radiation of these fish, but recent methodological advances, in particular in the field of geometric morphometrics, appear promising for answering a wide variety of evolutionary questions and even allow for addressing population-level questions regarding (adaptive) shape changes (reviewed in [160]).

In the present review, we only surveyed morphological studies. However, only the integration of evidence from various fields of research will significantly advance our understanding of the evolutionary mechanisms underlying the adaptive radiation(s) of (Lake Tanganyika) cichlid fish. For example, disruptive sexual selection on male colouration has been shown to drive speciation in some Lake Malawi and Victoria cichlids (e.g., [161–163]), and olfactory cues are important for mate choice in some Lake Malawi cichlids [164]. Thus, female choice for male nuptial colour and

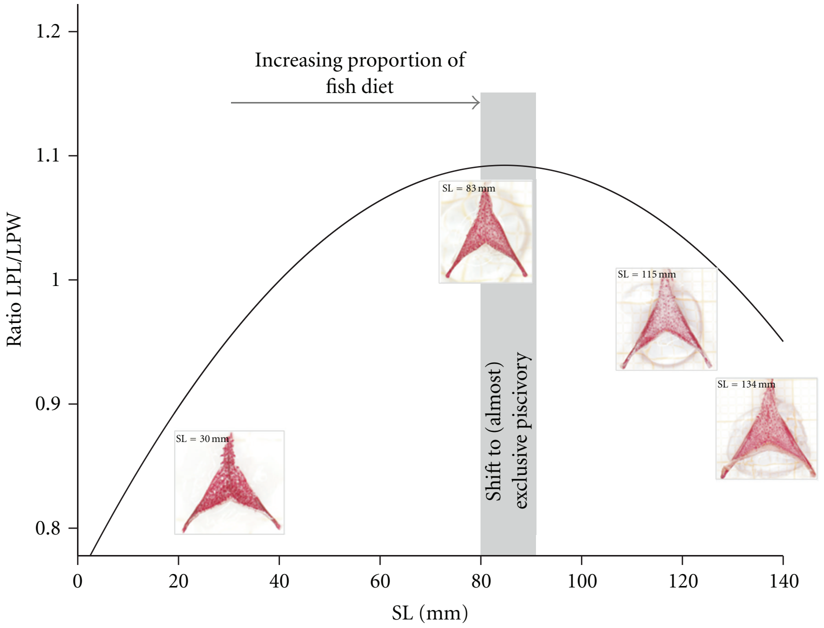

Figure 4: Schematic depiction of the allometric shape change of the lower pharyngeal jaw of *Lepidiolamprologus elongatus*, a diurnal top predator in Lake Tanganyika's shallow rocky habitat (modified from [70]). Note that the allometric shape change coincides with the shift to (almost) exclusive piscivory (data on feeding habits from [154–157]. The lower pharyngeal jaws of representatives of four distinct size classes are also shown to highlight the overall allometric shape change. SL, standard length; LPL, length of lower pharyngeal jaw; LPW, width of lower pharyngeal jaw.

olfactory signals may also have played (and still play) important roles in at least a part of the explosive radiation of the Lake Tanganyika cichlid species flock [58]. Indeed, more than 10% of Lake Tanganyika's cichlid species (e.g., Cyprichromini, Benthochromini, and some species of Ectodini, Tropheini, and Bathybatini) exhibit obvious sexual colour dimorphism [34]. However, at present there is no clear evidence for sexual selection based on body coloration being of great importance for driving rapid diversification in Lake Tanganyika's cichlid species flock. However, body coloration might serve as cue for species/mate recognition and prevent inter-specific or intermorph gene flow in case of secondary contact, be it human induced or due to lake level fluctuations, exemplified by evidence for colour assortative mating among originally allopatrically distributed colour morphs of *Tropheus moorii* [165–167]. Thus, allopatrically evolved mate choice cues serve as prezygotic isolation mechanisms preventing cichlid species/morphs from hybridization in the case of secondary contact.

Recent paleolimnological and geological studies have shed light on the dynamics of past water level fluctuations that act as "species pumps" by recurrent fragmentation and secondary admixis of populations [168] in the East African Great Lakes [169–171]. Ecological studies provide insights into the degree of intra- and inter-specific interactions (e.g., [172–179]), and the recent developments in sequencing techniques allow for the generation of huge amounts of sequence data at comparatively low costs provide exciting new possibilities to investigate phylogenetic relationships among taxa, population genetic structure, and the genetic basis and regulation of particular traits. At present, we are still far away from completely understanding what has driven and still drives the rapid diversification in East African cichlid species flocks, but recent advances in various fields of research hold a promising future for researchers.

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Review Article

The Utility of Geometric Morphometrics to Elucidate Pathways of Cichlid Fish Evolution

Michaela Kerschbaumer and Christian Sturmbauer

Department of Zoology, Karl-Franzens University of Graz, Universitätsplatz 2, 8010 Graz, Austria

Correspondence should be addressed to Michaela Kerschbaumer, michaela.kerschbaumer@uni-graz.at

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Fishes of the family Cichlidae are famous for their spectacular species flocks and therefore constitute a model system for the study of the pathways of adaptive radiation. Their radiation is connected to trophic specialization, manifested in dentition, head morphology, and body shape. Geometric morphometric methods have been established as efficient tools to quantify such differences in overall body shape or in particular morphological structures and meanwhile found wide application in evolutionary biology. As a common feature, these approaches define and analyze coordinates of anatomical landmarks, rather than traditional counts or measurements. Geometric morphometric methods have several merits compared to traditional morphometrics, particularly for the distinction and analysis of closely related entities. Cichlid evolutionary research benefits from the efficiency of data acquisition, the manifold opportunities of analyses, and the potential to visualize shape changes of those landmark-based methods. This paper briefly introduces to the concepts and methods of geometric morphometrics and presents a selection of publications where those techniques have been successfully applied to various aspects of cichlid fish diversification.

1. Introduction

Cichlid fishes are the most species-rich family of all teleost fishes, and their diversity is centered in the Great East African Lakes where more than 2000 species [1] evolved within the past five to six million years [2]. Consequently, the species assemblages of the three Great East African Lakes Victoria, Malawi, and Tanganyika represent the fastest vertebrate radiations known. Along with splitting into numerous species cichlid fishes have diversified into all imaginable ecological niches, manifested in spectacular diversification of the trophic apparatus. Cichlid fishes thus stand out as a paradigm for explosive speciation and adaptive radiation; their multitude of body shapes, specializations, colors, and behaviors is unsurpassed [3–10]. Their impressive ecomor-phological diversity is highlighted by the number of two-hundred-and-twenty recognized genera. The family Cichlidae has a wide distribution, spanning the southern tip of India and Sri Lanka with three species, Madagascar with 47 taxa in five genera (http://www.cichlid-forum.com/articles/species_list_madagascar.php), Central and South America with 400–500 species, and Africa with probably over 2500 species. Interestingly, cichlids have not colonized Australia. In accord with the sequence of the split of Gondwana, Indian and Malagasy cichlids form the most ancestral split in the diversification of cichlid fishes, followed by the split between the African and American lineages [11–15]. Only few species colonized North America and Asia (Jordan valley and Iran). The evolutionary age of cichlids is thus constrained by vicariance to 130–165 million years and the split between African and American cichlids to about 70–90 million years [16].

While riverine ecosystems tend to be relatively species-poor [17–19]—with the exception of the large South American rivers and perhaps the Zambezi system [20]—lakes comprise extremely species-rich communities. Their hotspot of biodiversity clearly lies in the three Great East African Lakes, Victoria, Malawi, and Tanganyika, where an estimate of 1800–2000 species, that is, 60% of the total cichlid diversity, are centered in species flocks [7]. Intralacustrine speciation has thus been put forward as the most common mode of diversification. Seehausen [21] listed 27 lacustrine radiations of cichlid fishes in Africa alone. As in all these lakes cichlid fish invariably form the most species-rich teleost lineage, they consistently outcompeted other fish groups when colonizing newly emerging lacustrine ecosystems [3].

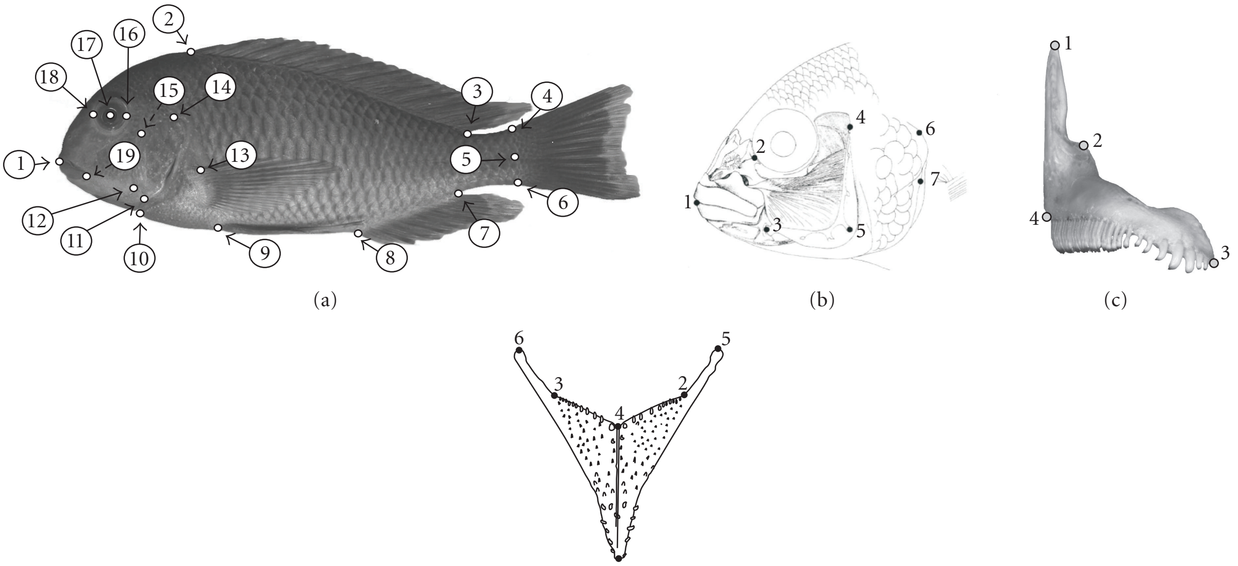
Adaptive radiation has been defined as the process of extremely rapid species formation coupled with ecological, morphological, and behavioral diversification [22]. The onset of adaptive radiation often requires two conditions to be met [23, 24]: (1) the formation of a new habitat or a dramatic change of an already existing habitat and (2) the possession of a key innovation, that is, a set of traits allowing for rapid adaptation towards novel niches. Thereby, one feature or a certain set of features allows one particular group to outrun the other taxa in the speciation and diversification process. In the case of East African lakes, several teleost groups had the same chance to colonize the newly emerged lakes: cyprinids, characins, mormyrids, various catfish, sardines, Nile perches, and spiny eels; but judging from endemicity rates, only cichlids underwent major diversification. In the case of cichlid fishes the key innovation may be a highly effective combination of factors: two sets of jaws (the oral jaws and the pharyngeal jaws; [25]) and a highly effective brood care system. The oral jaws deal with food acquisition and manipulation, and the pharyngeal jaws process food in a wide variety of ways before swallowing. Both jaws have evolved to realize diverse foraging strategies. Concerning brood care, cichlids invariably care for eggs and their young [4]. Ancestral lineages are substrate breeders from which several groups branched off by independently evolving various modes of mouthbrooding, that is, incubating the eggs and/or fry in the buccal cavity [26, 27]. Both brood care systems facilitate raising the young in densely packed communities.

Most cichlid species assemblages posed specific problems for comparative morphological methods: the accumulation of large numbers of closely related and morphologically similar taxa. It turned out that traditional morphometric methodology was pushed to its limitations and could not identify enough diagnostic characters to distinguish such closely related entities. Instead via de novo evolution of anatomical structures, cichlid diversification proceeded by modification of the same set of bones. New trophic niches were colonized by allometric changes together with shape modification of other body parts, so that cichlids could more efficiently adapt to novel food niches than other groups [28–30]. Despite clear ecological distinction many species are closely related and morphologically similar, and many diagnostic counts and measurements overlapped. This was the reason why geometric morphometrics was increasingly applied, often with dramatic success. It turned out that geometric morphometrics not only could address such subtle differences among individuals, populations, and species but also provided highly efficient means to assess multiple individuals in a quantitative way. Progress was also made in the selection and standardization of landmarks to avoid problems arising from potential influences from the subjectivity in placing particular landmarks. By stringent definition of particularly informative sets of landmarks, the body parts distinguishing the entities can be easily visualized and the underlying bony structures are identified.

By searching the current body of literature (Google Scholar, PubMed, British library, Scirus) we identified about 50 studies on cichlid fishes, applying the methods of geometric morphometrics (GM), hitherto. Seeded by D'Arcy Thompson's influential book on the mechanism of shape change published in 1917 [31], the discipline of geometric morphometrics started to become a widely used method in the early nineties [32, 33]. The first study focusing on cichlid fishes was published in 2001 [34]. This paper will first give a summary of GM methods with references of the comprehensive descriptions. It builds on a series of previous reviews [33, 35–42], but focuses on the cichlid fish model system and includes previous work by categorizing them into topical questions relevant for cichlid evolutionary approaches. Later we outline the opportunities to tackle important evolutionary questions via GM methods.

2. Overview on Geometric Morphometric Techniques

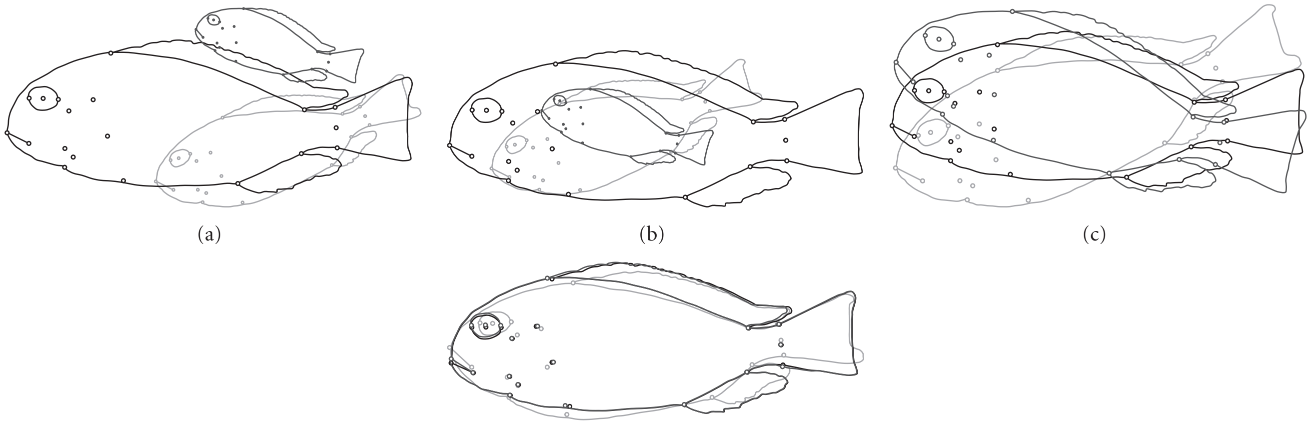
The term "morphometrics" is generally used for the statistical analysis of a large amount of distances, angles, or ratios of angles. "Geometric morphometrics" deals directly with coordinates of anatomical landmarks, either in two or three dimensions, rather than with traditional distance or angle measurements. Landmark points have been defined by Bookstein [32] as loci that have names as well as Cartesian coordinates. The names are intended to imply true homology (biological correspondence) from form to form. In Figure 1 we provide illustrations of different landmarks applied in different studies on cichlids. In addition to homologous landmarks there is the possibility to define the so-called semilandmarks [42, 46] that incorporate information about curves and outlines of the investigated objects. A considerable amount of methodological development has been achieved in recent years, as summarized by Gunz et al. [47]. Many morphological traits can be quantified by just measuring size of particular body parts, but there are other traits which are more complex and cannot be quantified by size alone. For such traits one needs the information about their shape. Shape is mathematically defined as the geometrical information of an object that remains when location, scale, and rotational effects are filtered [48]. The most common method to filter those effects and to compute shape coordinates is the so-called Procrustes superimposition [48]. The name Procrustes comes from Greek mythology; Procrustes physically attacked his guests (victims) by stretching them or cutting off their legs so as to make them fit an iron bed's size. In contrast to the mythological derivation of the term Procrustes in GM the relative positions of landmarks within a configuration are kept and actually do not get "fitted to an iron bed" during the alignment. Figure 2 describes and illustrates the steps of



(a) (b) (c)

(d)

Figure 1: Landmarks on (a) cichlid overall body (b) cichlid head [43] (c) a bony element (premaxilla) [44] and (d) the lower pharyngeal jaw [45].



(a) (b) (c)

(d)

Figure 2: Illustration of the Procrustes superimposition of the (a) original configuration (raw coordinates). (b) First, the centroid of each configuration is translated to the origin. (c) After that, configurations are scaled to a common unit size and (d) optimally rotated to minimize the squared differences between corresponding landmarks.

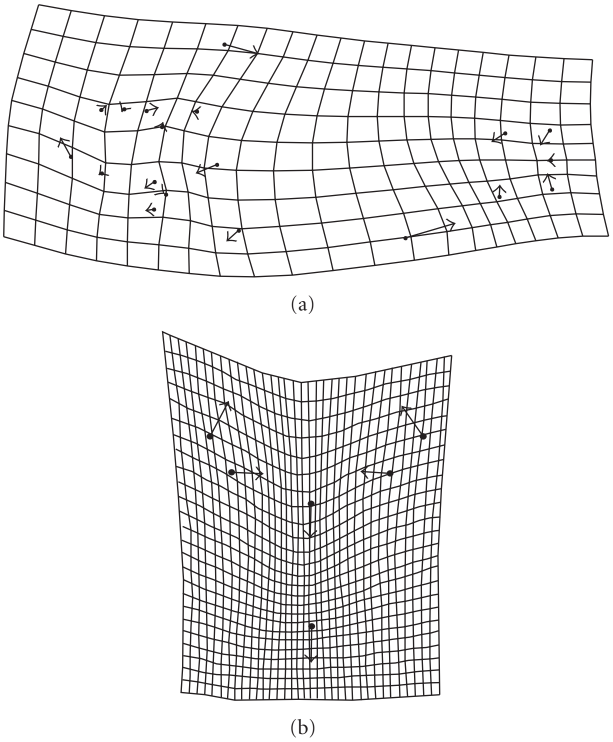
Procrustes superimposition. We note that there are several other accepted methods to obtain shape coordinates but the Procrustes method is the most prevalent one. Once shape coordinates, which are a reduced number of variables that collectively describe the variation in the original shapes, have been derived, results can be visualized and any type of statistical analysis can be performed.

There is a large body of free software for geometric morphometric analysis available on the internet. A comprehensive list of such software, much of it provided free of charge by various authors, is maintained by F. James Rohlf on the SUNY at Stony Brook morphometrics homepage (http://life.bio.sunysb.edu/morph/).

3. Application of GM to the Study of Cichlid Evolution

3.1. Addressing Cichlid Diversity—How and to Which Extent Do Groups of Cichlids Differ in Shape?

Geometric morphometrics provide the opportunity to get new insights in the variety of morphological characteristics, discriminating genera, species, populations, and morphs or even individuals. Kassam et al. [49] compared groups of cichlids from different African lakes. They used landmark-based geometric morphometric methods to test the hypothesis that *Petrochromis* species from Lake Tanganyika are ecomorphologically equivalent to *Petrotilapia* species from Lake Malawi. The study identified differences between the lakes, but demonstrated morphological equivalence for three species and hence their convergent evolution. In another interlake comparison published in 2007, Kassam et al. [50] emphasized the robustness of GM when testing the morphological equivalence hypothesis among cichlid species. A third study addressed the variation in body shape of the Central American *Amphilophus citrinellus* species complex from lakes of Nicaragua by means of GM [51]. In the same year, Parsons et al. [52] compared the results of several traditional morphometric analyses against geometric morphometric analyses. He applied traditional and geometric morphometric methods to a common data set, namely, two Nicaraguan species, and thereby showed that GM is the more effective method to analyze and interpret body form. This message was also given by Trapani [53], who compared dental morphs of cichlids and by Maderbacher et al. [54], where traditional and geometric morphometric methods were compared to discriminate populations of cichlids. Postl et al. [44] showed that GM is possible to discriminate even populations of the Tanganyikan cichlid *Tropheus moorii*, which was presumed not to differ in morphology but only in coloration. All mentioned studies have in common that they benefited from the fact that GM is not constrained by focusing on particular shape features a priori, so that it was possible to indentify differences in any direction of shape space. Such shape differences among groups can be easily visualized through deformation grids in the so-called thin-plate splines (see Figure 3).


(a)

(b)

Figure 3: Deformation grid or Thin-plate spline showing differences (a) concerning whole body shape (19 landmarks, see Figure 1(a)) between a group of cichlid individuals living in the natural environment in Lake Tanganyika and another group of fish raised in ponds with a standardized rock architecture and artificial food. The grid accords to a canonical variate analysis carried out in an unpublished study on Lake Tanganyikan cichlids. (b) of the lower pharyngeal jaw throughout ontogeny between the smallest and the largest individual of *Lepidiolamprologus elongatus* (6 landmarks, see Figure 1(d)).

3.2. Trophic Ecology—What Do Differences/Similarities in Trophic Morphology Tell Us about Cichlids Evolution?

Cichlid fishes exhibit a wide variety of feeding specializations, which appear to be strongly associated with adaptive changes in trophic morphology. So it stands to a reason that researchers quantify differences in trophic structures to obtain new insights into pathways of cichlid diversification. In 2001, Rüber and Adams [34] were the first to apply GM in a cichlid fish evolutionary study. By quantifying morphological variation in body shape and trophically associated traits among taxa of the endemic Lake Tanganyika tribe Eretmodini, they could argue that similar body shape and feeding strategies evolved multiple times in independent lineages, even within a single tribe of cichlids. Indirectly they demonstrated that body shape was not fully independent of trophic morphology. In another study on cichlid eco-morphology Bouton et al. [43] demonstrated that in haplochromine cichlids head shape varies between populations at rocky islands at distances of only kilometers apart and that this variation—possibly in response to environmental variables—may lead to allopatric divergence of rock-dwelling cichlids (the landmark set from this study is shown in Figure 1). In particular, morphology of oral and pharyngeal jaws was frequently investigated with GM methods. In one of those studies Albertson and Kocher [55] analyzed four skeletal elements of the oral jaws in two closely related species from Lake Malawi and their F_1 hybrid progeny. Because hybrids were significantly different from both parental species they suggested an additive mode of action for the alleles in question. This work formed the basis for three consecutive studies to further elucidate the genetic architecture of the cichlid jaw apparatus [56–58].

Kassam et al. [59] quantified the shape of several bony elements from head region and compared patterns of variation within and between zooplankton and algal feeders of cichlid fishes. They were able to distinguish among those groups, but also revealed trophic morphological variation between species within each trophic guild. Other studies showed that GM is not only applicable to quantify tooth shape in the oral jaw [53, 60] but is also an adequate method to investigate differences in pharyngeal jaws [45, 61, 62]. These studies confirmed that this structure is a highly adaptive morphological feature which is optimized for processing several different types of food in cichlid fishes.

It is worth mentioning that cichlid pharyngeal jaws were one of the first examples of object symmetry in geometric morphometrics [63]. They played an important role in developing new morphometrics methods studying symmetry and asymmetry. Those methods are available in specific "canned" software such as the specialized Sage program [64] and the more general MorphoJ package [65] and could be used in future studies concerning different topics in cichlid research for example, asymmetry in scale-eating cichlids [66].

3.3. *Growth Allometry—At What Time and for What Reason Do Morphological Differences Arise during Growth of a Cichlid?*

Since 1924 it has been established that morphological adaptation can proceed via allometry, the change in relative dimensions of body parts that are correlated with changes in overall size [31, 67]. Gould [68, 69] emphasized that changing size often means changing shape. In practice, such allometric relations can be studied during the growth of a single cichlid individual, between different individuals within one species or between different species. Fujimura and Okada [70] compared the shapes of the lower jaw bone during different stages of development of a Lake Victoria cichlid and a riverine cichlid using GM methods. Their data suggested that most shape change may have a similar pattern during the growth of both species. Differences in adult shape may be due to differences arising early in development, and not to the difference of shape change during growth. A very recent study [45] described allometric shape change of the lower pharyngeal jaw in a Lake Tanganyika cichlid fish and could correlate those shape changes to a dietary shift in ontogeny of this species. In Figure 3(b), which originated from the work of Hellig et al. [45], it becomes clear that illustration of results is one great advantage of GM.

Another topic often linked to that about allometry is sexual dimorphism. There are several studies dealing with sexual size dimorphism in cichlids [71–74] but only few authors have addressed shape dimorphism by applying GM methods. We found two studies where sexual dimorphism was touched on within another topic [54, 75] and one recent study by Herler et al. [76] where sexual dimorphism was investigated through geometric morphometrics and shape differences among sexes were assessed in relation to the differentiation of populations and species in a Lake Tanganyika cichlid genus.

3.4. Geometric Morphometrics as Valuable Tool in Integrative Evolutionary Studies on Cichlid Fishes. The following studies combined several approaches to address evolutionary questions. The first is meanwhile highly cited and presented a convincing case of sympatric speciation in the Midas cichlid species complex (*Amphilophus* spp.) in a crater lake in Nicaragua [61]. This study included phylogeographic, population-genetic (based on mitochondrial DNA, microsatellites, and amplified fragment length polymorphisms), ecological, and morphometric analyses, whereby morphometric analyses integrated quantification of body shape and shape of pharyngeal jaws by means of GM. Another integrative evolutionary study by Stewart and Albertson [77] focused on Lake Tanganyika scale-eating cichlids which show a frequency-balanced polymorphism in the left- and right-handedness of the mouth, as adaptation to scale-eating [78]. They addressed the evolution and development of craniofacial morphology and laterality in these cichlids. Indeed, their data supported a genetic basis for jaw laterality. They observed jaw laterality early in development and identified a conserved locus segregating with craniofacial handedness in East African cichlids.

Two other recent studies compared cichlids from the three East African lakes. The most recent from Cooper et al. [79] dissected specimens from 87 genera of cichlid fishes and compared head shapes and underlying structures using geometric morphometrics. The authors confirmed that specific changes in trophic morphology have evolved repeatedly in the African rift lakes. One year before another study on African cichlids and their adaptive radiation was published where GM methods played an important role. Young et al. [80] introduced a modified methodology of Principal Component Analysis (PCA) combined with a linear regression, called "ordered-axis plot." This novel approach is a powerful tool to detect patterns of divergence among separate groups in a common morphospace [80], although Parsons et al. [81] pointed out the limitations of this method in the application to general data sets.

Another comprehensive study on cichlid fishes, carried out by Stelkens et al. 2009 [82], produced interspecific F_1-crosses of two African haplochromine cichlid fish species with a particularly large degree of pairwise genetic and phenotypic divergence. The transgression in multitrait shape phenotypes was quantified using GM methods. They concluded that hybridization may generate novel genotypes with adaptive potential and that interspecific hybridization has likely contributed to the rapid rate of phenotypic evolution in the adaptive radiations of haplochromine cichlids.

4. Conclusions and Outlook

To date about 50 publications have accumulated with a focus on cichlid fish adaptive evolution and adaptive radiation. Clearly, geometric morphometric methods are gaining importance in cichlid research, and several advantages became evident. These begin with the efficiency of data acquisition and end with the impressive potential for visualization of the results. Geometric morphometrics can be based on photographs and even computer scans of anesthetized fish, so that sacrificing and preserving of specimens is unnecessary, unless voucher specimens are needed or the investigated body parts require the dissection of an individual. The positioning of landmarks can be individually adapted to particular research questions, so that GM methods can be broadly applied for a wide variety of evolutionary questions involving complex shape changes. The sensitivity turned out to be high enough to address population-level questions involving (adaptive) shape changes. In contrast, traditional comparative morphological approaches are often based on selected measurements, and results are somewhat restricted to those few variables.

Other exciting topics with great potential would be the analysis of phenotypic plasticity and its influence on diversification, the genetics of adaptation and ecological speciation in conjunction with new-generation sequencing approaches or to study the modularity in the trophic apparatus. Allometric changes during growth might also bear great potential. Another issue is that all GM studies on cichlids were based upon 2-dimensional data. We are convinced that 3-dimensional data will rapidly become accessible in cichlid research, given the rapid progress in the field. To conclude, there are several promising trajectories to address important evolutionary questions via GM, so that there is no doubt that this field will evolve further rapidly.

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Research Article

Modularity of the Oral Jaws Is Linked to Repeated Changes in the Craniofacial Shape of African Cichlids

Kevin J. Parsons, W. James Cooper, and R. Craig Albertson

Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA

Correspondence should be addressed to Kevin J. Parsons, kjpars01@syr.edu

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The African cichlids of the East-African rift-lakes provide one of the most dramatic examples of adaptive radiation known. It has long been thought that functional decoupling of the oral and pharyngeal jaws in cichlids has facilitated their explosive evolution. Recent research has also shown that craniofacial evolution from radiations in lakes Victoria, Malawi, and Tanganyika has occurred along a shared primary axis of shape divergence, whereby the preorbital region of the skull changes in a manner that is, relatively independent from other head regions. We predicted that the preorbital region would comprise a variational module and used an extensive dataset from each lake that allowed us to test this prediction using a model selection approach. Our findings supported the presence of a preorbital module across all lakes, within each lake, and for Malawi, within sand and rock-dwelling clades. However, while a preorbital module was consistently present, notable differences were also observed among groups. Of particular interest, a negative association between patterns of variational modularity was observed between the sand and rock-dwelling clades, a patter consistent with character displacement. These findings provide the basis for further experimental research involving the determination of the developmental and genetic bases of these patterns of modularity.

1. Introduction

Adaptive divergence is likely influenced by the coordination and integration of multiple traits. If genetic variation affecting patterns of trait covariation have fitness consequences, then a particular pattern of integration that allows for a closer match to a new local multivariate phenotypic optimum should be favoured [1–3]. Alternatively, ancestrally conserved patterns of integration may act to constrain the rate and direction of evolution by preventing certain functions from evolving [4, 5]. Either way, modularity may influence the pace of evolution and determine evolvability [6, 7]. It is therefore not surprising that the study of trait integration has been of interest to biologists for more than half a century [8–10] and has recently seen renewed attention [3, 11–14].

The study of integration has more recently been extended to the closely related concept of modularity—the relative degrees of connectivity in systems. A module is a tightly integrated unit that is relatively independent from other such modules. For morphological data, modularity has been studied in a variety of contexts including those that are developmental, genetic, functional, and evolutionary in their focus [15–22]. An emerging consensus is that patterns of modularity in complex phenotypes likely represents a balance between functional and developmental integration and that modularity is better viewed as a matter of degrees rather than an all-or-nothing phenomenon [12].

It has been suggested that modularity can facilitate divergence by allowing organisms to alter aspects of their phenotype without facing the developmental or fitness tradeoffs that would be present in a wholly integrated unit [12, 13]. In this way, the evolution of modularity could be tied to the idea of key innovations (see [23] for an example). The origin or evolutionary “success” of taxa is often attributed to key innovations—aspects of organismal phenotype that promote diversification [24]. Key innovations may enhance competitive ability, relax adaptive tradeoffs, or permit exploitation of a new productive resource base.

The African cichlids from lakes Victoria, Tanganyika, and Malawi in East-Africa’s Great Rift Valley represent the largest extant example of vertebrate adaptive radiations known [25]. Certain anatomical features of this group have been proposed as key innovations that have facilitated the rapid evolution of these fishes [25, 26]. The best known of these represents an important example of functional modularity, wherein the highly derived cichlid pharyngeal jaw mechanism allows the processing of prey within the throat to be decoupled from prey capture by the oral jaws [12, 26]. This is thought to have allowed African cichlids to exploit a wide array of niches that would be unavailable if only one set of jaws was present [27].

The cichlid radiation of Lake Malawi is particularly interesting, because although it is intermediate to Tanganyika and Victoria in terms of age and morphological diversity [28, 34], it has produced the greatest number of endemic species (well over 700) [35, 36]. The evolutionary history of Malawi cichlids suggests that current diversity arose via three stages of diversification: (1) early divergence of the sand-dweller and rock-dweller clades, each of which has adapted to a major macrohabitat, (2) competition for trophic resources within each of these clades that caused further differentiation of trophic morphology, and (3) divergent sexual selection resulting in differentiation of male nuptial coloration [37, 38].

We recently completed an extensive analysis that explored patterns of craniofacial shape variation in African cichlids from each of the three rift lakes [28]. Our data, which represented approximately 80% of the genera across lakes, revealed that all three cichlid radiations share a common trajectory of divergence with respect to each lineage's major axis of divergence (PC1). Our geometric morphometric analysis also showed that these changes were primarily related to changes in the relative length and size of the "preorbital region" of the skull, which encompasses the oral jaws and supporting structures, with shape posterior to the orbital region remaining relatively stable. These trends suggest that a large portion of the head diversity seen in African cichlids has been achieved by relatively simple and repeated shifts in jaw shape and that these may have happened relatively early in their evolutionary history.

Functional differences in jaw size reflect divergent foraging modes. African cichlids with longer oral jaws are either "suction feeders" and forage on zooplankton, or they are piscivorous and feed on other fishes [28]. Alternatively, species with shortened jaws are typically "biters" that possess a higher mechanical advantage to scrape algae or forage on large macrobenthic prey. In Lake Malawi, this fundamental division is reflected in the cladogenic split between rock- and sand-dwelling species. On average, rock-dwelling species have a shorter jaw in common morphospace, whereas sand-dwellers species have relatively longer jaws [28, Cooper unpublished data]. Notably, these morphological patterns seem to be a common theme in the adaptive radiation of other fish assemblages (e.g., [39]) and even in population-level divergence among ecomorphs of charr whitefish, and sunfish [40–42]. Thus, a propensity for changes in the size of oral jaws seems to exist in teleosts at multiple levels of biological organization and perhaps represents a key innovation for this group as a whole. While the evolutionary origins for a preorbital module may not lie within African cichlids examining potential patterns of craniofacial modularity in cichlids may identify important targets for future developmental genetic research to understand the proximate mechanisms that have facilitated these important radiations and divergence in other groups of fishes. Cichlids may be especially useful for this research, because species with widely variable jaw morphologies can be hybridized, facilitating the creation of large populations for genetic mapping to identify the loci and genetic pathways that underlie changes in jaw shape [43, 44].

As mentioned, Liem's [26] seminal work on the pharyngeal jaw apparatus in cichlids suggested that the functional decoupling of prey capture and processing should free the oral jaws to more readily adopt an array of niche-specific shapes for food capture, largely independent of other traits. Implicitly, this insight confers a level of modularity to the cichlid oral jaw apparatus. Recent work in our lab, as well as from others, supports this assertion by demonstrating that morphological divergence among rift lake cichlids is characterized by prodigious shifts in oral jaw shape [28, 34] and has lead to the specific hypothesis that the preorbital region of the skull represents an evolutionary module that is conserved among cichlids from each of the three East African rift lakes [28]. Here, we objectively test this hypothesis by comparing multiple combinations of models of cichlid head variational modularity. Specifically, we use an approach of model selection recently introduced by Márquez [45] to statistically assess patterns of variational modularity across a large sample of rift valley lake cichlids. To determine whether similar patterns of modularity are operating at different levels of biological organization, we also examine craniofacial modularity in each lake separately, as well as within the rock- and sand-dwelling clades of Lake Malawi.

2. Materials and Methods

2.1. Data Collection. The data used for this study has been previously published in Cooper et al. [28], where further details, including a full list of specimens sampled, can be found. Briefly, our sampling included 78.8% of the genera endemic to the three East-African rift lakes, with the following percentages from each lake: Tanganyika (74.5%), Malawi (88.5%), and Victoria (57.1%). Within Lake Malawi, 19 rock-dwelling species, representing 11 genera, were sampled, and 36 sand-dwelling species, representing 31 genera, were also sampled. Dissections were performed on cichlid heads in order to expose anatomical landmarks important for oral jaw function (Figure 1). A total of sixteen anatomical landmarks were plotted on the images of each specimen using the software program tpsDig2 [46].

2.2. A Priori Hypotheses. Our goals were to determine first whether modularity was present in the cichlid head and second what the best-supported pattern of modularity was in our data. This required comparative testing of alternative *a priori* models, each of which specified a particular modular structure in the cichlid head. In this approach, each model is comprised of a series of partitions defined as anatomical

TABLE 1: *A priori* hypotheses of modularity in the cichlid head. Brackets denote putative modules. Note that two similar models are presented for jaw function.

Model	Description
[1,2,3,4,5,6,9,10,11] [7,8,12,13,14,15,16]	Preorbital/postorbital—major evolutionary trends in cichlids suggest that the preorbital region can independently change shape during adaptive radiations in African cichlids [28]
[1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16]	Full integration—the entire head operates as a completely integrated unit, and this hypothesis also compliments the existing null hypothesis of no integration
[1,3,4,11,15] [2,5,6,16] [13,14,15] [7,8,9,10,12,13] [1,3,4,5,11,15] [2,5,6,16] [13,14,15] [7,8,9,10,12,13]	Jaw function—modules are parsed on the basis of functional uses and the attachment of muscle and ligament—[29–32] [29, 34]
[13,14,7] [8,9,10] [1,2,3,4,5,6,11,12,15,16]	Respiration/vision/biting—modules are parsed based on their primary functional roles

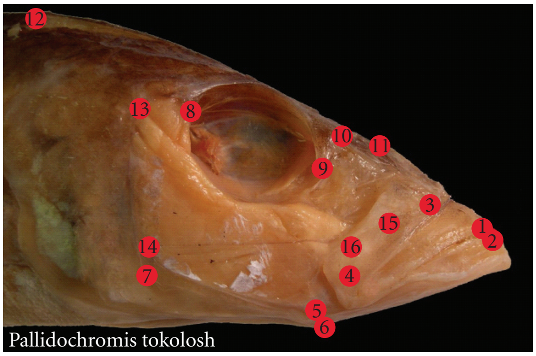

FIGURE 1: Anatomical landmarks placed on the cichlid head. 1 = Tip of the anterior-most tooth on the premaxilla; 2 = Tip of the anterior-most tooth on the dentary; 3 = Maxillary-palatine joint (upper rotation point of the maxilla); 4 = Maxillary-articular joint (lower point of rotation of the maxilla); 5 = Articular-quadrate joint (lower jaw joint); 6 = Insertion of the interopercular ligament on the articular (point at which mouth opening forces are applied); 7 = Posterio-ventral corner of the preopercular; 8 = Most posteriorventral point of the eye socket; 9 = The most anterioventral point of the eye socket; 10 = Joint between the nasal bone and the neurocranium; 11 = Posterior tip of the ascending process of the premaxilla; 12 = Dorsal-most tip of the supraoccipital crest on the neurocranium; 13 = Most dorsal point on the origin of the A1 division of the *adductor mandibulae* jaw closing muscle on the preopercular; 14 = Most dorsal point on the origin of the A12 division of the *adductor mandibulae* jaw closing muscle on the preopercular; 15 = Insertion of the A1 division of the *adductor mandibulae* on the maxilla; 16 = Insertion of the A2 division of the *adductor mandibulae* on the articular process.

regions delimited by landmarks, each representing a hypothesized module predicted to be highly integrated relative to other such partitions.

Based on knowledge of the development and biomechanical function of cichlid heads, we constructed a number of hypotheses of modularity that were intended to extensively cover potential patterns of covariance. We selected a total of five *a priori* models representing the spatial distribution of developmental units and functional components of the cichlid head (see Table 1). An additional "null" model representing a lack of any integration or modularity was included in our analyses. Because it is not biologically realistic to expect that patterns of modularity predicted by these developmental and functional models are mutually exclusive, all possible nonnested combinations of the modules defined by the original five hypotheses of modularity were also included in model comparisons. In total, 137 competing models were tested. It is important to note that while this list of hypotheses is far from exhaustive, it represents an extensive collection of models—likely covers a substantial proportion of the developmental and functional processes capable of affecting covariation in the cichlid head.

2.3. Modularity Analysis. The methodology for testing *a priori* hypotheses of modularity was adapted from an approach proposed by Márquez [45] consisting of four basic steps implemented in the Mint software package (available at: http://www-personal.umich.edu/~emarquez/morph/).

(1) Computation of an expected covariance matrix from each model of modularity, by assuming that each module resides in its own subspace within the phenotypic space occupied by the entire structure, as described in Márquez [45].

(2) Computation of a goodness of fit statistic, γ, to measure the dissimilarity between observed and expected covariances for each model, as

$$\gamma = \operatorname{trace}\left\{(\mathbf{S}-\mathbf{S}_0)(\mathbf{S}-\mathbf{S}_0)^T\right\}, \tag{1}$$

where $\mathbf{S}$ and $\mathbf{S}_0$ are the observed and modeled covariance matrices and T is the transpose symbol [47]. To ensure the comparability of this statistic across models, γ is standardized twice: first, all γ values are divided by $\gamma_{\max}$, corresponding to the null model describing complete absence of integration, so that γ is scaled to vary within the interval [0, 1]; second, scaled γ is standardized via linear regression to remove the effect of the number of estimated parameters in models, which takes advantage of the linear relation observed between γ and the number of zeros in models.

The standardized statistic is defined as the residual m $\gamma^* = \gamma - f(z)$, where $f(z)$ represents the linear function relating the values of γ computed from all possible models of modularity to their corresponding counts of zero elements, z. Even though it would be computationally unfeasible for most studies to include all possible models, the fact that scaled γ values are restricted to the interval [0, 1], where 0 corresponds to the observed covariance matrix and 1 to the null model of no integration, implies that $f(z)$ must also vary within these limits, which are sufficient to define the linear function for any given set of variables. Given a large random sample of models, with γ values symmetrically distributed about their mean, $E(\gamma) - f(z)$, and thus $E(\gamma^*) - 0$. Consequently, models in which $\gamma^* < 0$ correspond to comparisons where observed covariances are relatively low on average and hypothesized to be zero, and conversely, cases where $\gamma^* > 0$ occur when relatively high covariances are on average hypothesized to be zero, the best-fitting model is that with the lowest γ^* value. Note that this approach differs slightly from the one used in Márquez [45], where $f(z)$ was estimated via regression using only the models included in a study, as opposed to all possible models. 95% confidence intervals were computed as the 2.5 and 97.5 percentiles of a distribution of 1,000 jackknife subsamples [48] formed by removing random subsets of 10% of the specimens from each sample.

(3) The statistical significance of γ^* was assessed using a parametric Monte Carlo approach. In these tests, a null distribution for the statistic is generated by comparing the original observed covariance matrix **S** to each of 1,000 random matrices generated from a Wishart distribution with mean vector **0** (i.e., the same mean as Procrustes residual data) and covariance matrix **S** [45, 49].

(4) Finally, to allow choice among the multiple models that are significantly better than chance according to the Monte Carlo approach described above, models are ranked by their goodness of fit (i.e., γ^* values, in ascending order). The relative support for each model is determined by computing the stability of its rank using a jackknife approach in which γ^* values and model ranks are recomputed after removing a random portion of the samples. In this study, we removed 10% of the data in each of 1,000 jackknife replicates.

2.4. Comparisons of Covariance among Lakes, Sand Dwellers, and Rock Dwellers. If a single model fits two of our groups (Lake Tanganyika, LT; Lake Malawi, LM; Lake Victoria, LV; Rock dwellers, RD; Sand dwellers, SD) equally well, it would not necessarily mean that they were close to each other in our model space. This is because two objects that are equally distant from a third (the best supported model) are not required to occupy the same position, especially in a high-dimensional space. In our case, the γ^* values calculated for each group represents reference points useful for determining their relative position. This vector of γ^* values can have two interpretations, the first as a set of distances between the observed covariation matrix and known patterns of modularity and the second as coordinates for the data in "model space" centered on a group covariance pattern. Because each group may be centered at a different position, only the direction of these vectors can be compared, which was achieved through the use of correlations between γ^* vectors of each group. This involved two separate analysis; first, we determined levels of correlation for γ^* across the three lakes; second, we determined levels of correlation for γ^* among LM, RD, and SD groups. However, we did not use all 137 possible gamma values in these correlations; rather, we used the ten top-ranked models in for each group. This increased the likelihood that we were testing associations between the most biologically relevant models.

3. Results

Monte Carlo tests were unable to distinguish among models, suggesting that hypotheses were too similar to distinguish amongst each other given available sample sizes. We, therefore, focus our interpretations on the basis of the relative rankings of γ^* values and their jackknife support.

Overall, there was strong support for the hypothesis that modularity is present in the heads of African cichlids. Across the three lakes the null model of no integration was ranked 57th, 100th, and 102nd, out the 137 models in LV, LM, and LT, respectively. In the RD and SD groups the null model of no integration ranked 70th and 108th, respectively. Jackknife tests provided high support for these rankings in all groups.

3.1. Top-Ranked Models. At all levels, the best supported hypothesis included one preorbital and one postorbital module. In our pooled data set across all lakes, as well as separate data sets for LV and RD, a preorbital module that defined the upper jaws and encompassed the exact same set of landmarks was identified (Figure 2). Support for these patterns of modularity was high with the top model in the pooled sample of cichlids being ranked number 1 in 96.6% of jackknife reps. LV and RD groupings had top models that were similarly highly supported with 84%, and 85% of jackknife reps, respectively.

For LM as a whole and the SD sample, the top ranked models displayed a preorbital module that encompassed both the upper and lower jaws (Figure 2). Statistical support for the LM model was high with 86% of jackknife reps maintaining its top ranking. In the case of the SD sample, there were two, statistically indistinguishable top models: The highest-ranked model included three modules, one encompassing the oral jaws, one defining the orbital size, and another that covers much of the posterior region of the head. The second ranked model was identical to the first with the exception that it did not possess an eye/orbital module. Support for the best SD model (i.e., three modules) was low, with only 47% of jackknife reps supporting its ranking. The second best SD model (i.e., two modules) was also ranked as the best model in 44% of jackknife reps. However, a subsequent set of analyses found that when one of these models was removed, support for the other model significantly improved to where its top ranking was supported in over 97% of jackknife reps. This analysis suggests that both models are equally valid.

The LT dataset also showed strong support for a preorbital module in its top-ranked hypothesis (supported in 98.6% of jackknife reps). However, it differed from the other groups by having a preorbital module comprised primarily of the lower jaw (Figure 2).

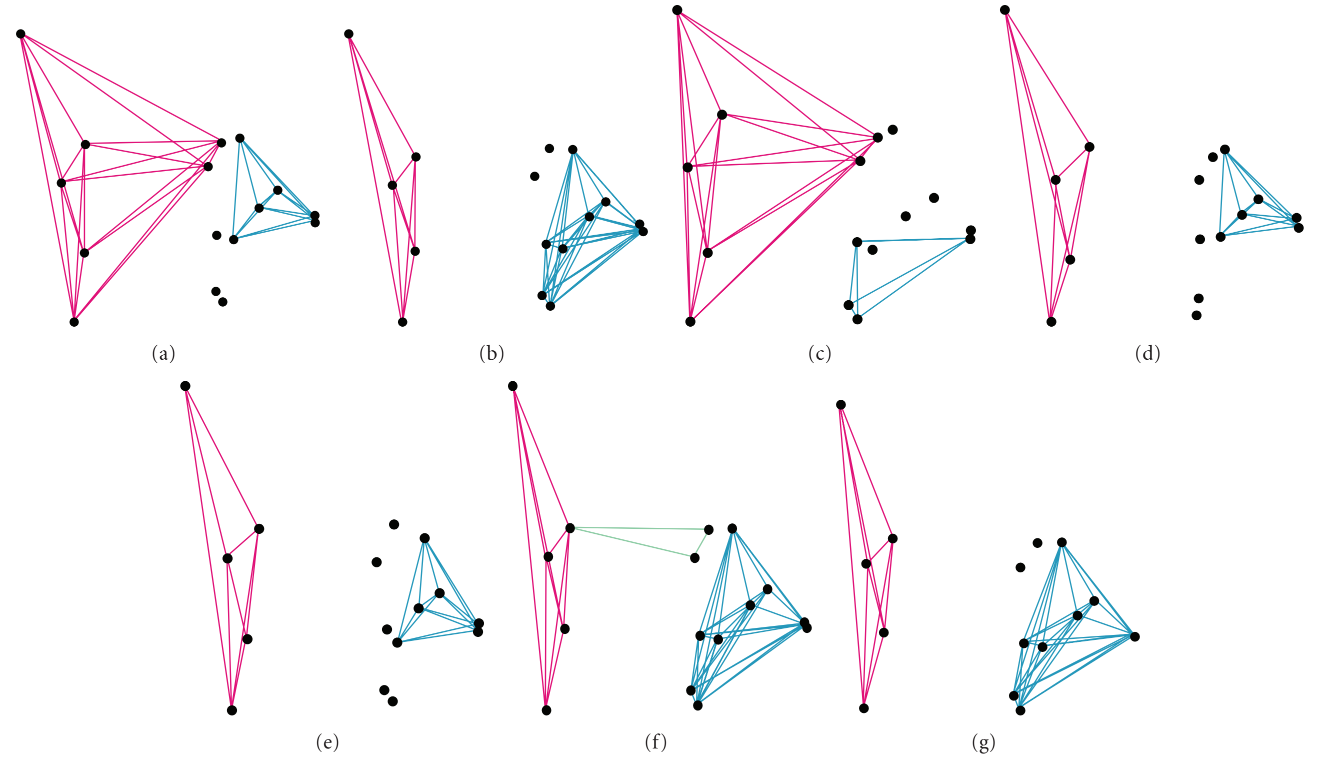

Figure 2: The best supported hypotheses of modularity for African cichlids as a whole (a) and their respective adaptive radiations within lakes Malawi (b), Tanganyika (c), and Victoria (d). Also shown are the best-supported patterns for rock (e), and sand dwellers (f, g) within Malawi. Two models are shown for rock dwellers, because our statistical analysis was unable to discern whether one hypotheses was significantly better at describing patterns of covariance. Note that despite differences among these hypothesis, each contains a preorbital module based in the oral jaws.

Table 2: Correlation coefficients of γ^* values for the top ten ranked hypotheses of modularity between cichlids in Malawi, Tanganyika, and Victoria.

	Malawi	Tanganyika	Victoria
Malawi	1.000	—	—
Tanganyika	0.974	1.000	—
Victoria	0.991	0.969	1.000

Table 3: Correlation coefficients of γ^* values for the top ten ranked hypotheses of modularity between cichlids in Malawi as a whole, as well as the rock- and sand-dweller division within the lake.

	Malawi	Rock dwellers	Sand dwellers
Malawi	1.000	—	—
Rock dwellers	−0.027	1.000	—
Sand dwellers	0.784	−0.494	1.000

3.2. Relationships among Patterns of Covariance Across Lakes, and between the Lake Malawi Sand and Rock Dwellers. Across the three lakes, we observed strikingly similar patterns of covariation. We used γ^* values from a total of 23 hypotheses of modularity, reflecting the top ten ranked models for each of the three lakes, meaning that 7 of these hypotheses were shared among lakes. The r-values for our tests were all extremely high, and positively correlated, indicating that in spite of differences between top-ranked models, very similar patterns of covariance underlie each of these adaptive radiations (Table 2).

We also found that patterns of covariance may be diverging within LM. We used a total of 18 models to describe the top ten models across LM and within the RD and SD datasets. Thus, a total of 12 out of a possible 30 models were shared among these groups. The correlation between γ^* values for LM as a whole and SD dataset was particularly strong, indicating that sand dwellers may be influencing the overall pattern of modularity exhibited by Malawi cichlids. This result could be due, in part, to their larger relative sample size compared to RD cichlids. Alternatively, LM as a whole showed almost no relationship with RD species, and there was a strong negative relationship between SD and RD species (Table 3). These data suggest that patterns of trait covariance are being repelled between SD and RD.

4. Discussion

Our results demonstrate that a preorbital module is present in the oral jaws of East African rift valley cichlids and that this pattern of covariation is conserved across all lakes. This trend strongly supports the hypothesis that this pattern of modularity has influenced the rate and direction of adaptive phenotypic divergence among African cichlid radiations—an idea rooted in the proposal that the cichlid pharyngeal jaw apparatus is a key innovation that freed the oral jaws from a functional constraint [26], formalized in light of quantitative patterns of trophic divergence among cichlid lineages [28], and empirically tested here. While our results are compelling, we suggest that the comparisons of rates of evolution to other groups which lack a pharyngeal jaw apparatus (e.g., salmonids and characids), and possibly a preorbital module, may be needed to confirm whether the patterns of modularity identified in cichlids represent a key innovation.

4.1. Conserved and Divergent Patterns of Craniofacial Modularity among Cichlids. While the results of our correlation analyses indicate that general patterns of covariance are conserved across lakes, there were several notable differences in the top-ranked hypotheses of modularity among groups, suggesting that while conserved patterns exist, modularity itself is capable of evolving. The LM dataset had a pattern of modularity in which the preorbital module encompassed both the upper and lower jaws, while in the LT dataset, the preorbital module was exclusive to the mandible. For the LV dataset, integration was most prevalent for the upper jaws. The LV radiation is the youngest of the three rift lakes [50], and correspondingly, our prior analysis found relatively low levels of shape variation (disparity) in this lake compared to LM and LT cichlids [28]. Also, more than 60% of the morphological variation among species in LV can be explained by a single major axis (principal component), considerably more than was explained by this shared axis for LM and LT (i.e., Victoria cichlid head anatomies were relatively more integrated). Taken together, these results suggest that the younger divergence in LV is determined by a more limited set of strong interactions among traits. Since the upper jaw contains the anatomical linkages most responsible for highly kinetic jaw movements, such as jaw protrusion, this would imply that both the functional and morphological evolution of this lineage has been constrained. As the youngest of the three rift lake lineages, patterns observed within LV may offer insight into the proximate mechanisms that have shaped cichlid radiations in general. It is possible that the pattern of modularity we have identified in LV has played a dominant role in the early patterns of divergence of cichlids in LM and LT. Consistent with this idea, the preorbital module identified in the upper jaw for LV was very similar to one identified in the top-ranked model for our pooled data set across all cichlids (Figure 2).

The top-ranked models for the SD and RD clades within LM also exhibited notable differences. Whereas the SD group exhibited a preorbital module that included both the upper and lower jaws, RD species expressed a pattern of modularity similar to that of LV, where only the upper jaws were integrated. Moreover, the SD/RD division within Malawi was characterized by a strong negative relationship in covariance patterns, suggesting that ecological competition between these clades during the early history of the lake may have caused patterns of trait covariance to diverge. This pattern is consistent with character displacement, but at a different biological scale (groups of species or clades) than where it is usually recognized [51–53]. Character displacement is often thought to occur between two closely related species; however, research suggests that character displacement can also occur between distantly related species, as well as whole communities [54, 55]; see also [56, 57] for evidence of character displacement in African cichlids. Therefore, it is appropriate to speculate that this process is contributing to divergence between SD and RD clades in LM.

Integration between the upper and lower jaws, as displayed by the SD dataset, may be especially advantageous for ram/suction-feeding predators, a predominant SD trophic niche [58], because both jaws need to work together in a highly coordinated fashion to produce kinematic force [59]. Alternatively, in RD species that most often employ a biting tactic whereby the upper jaw is relatively more stationary during foraging [26], the upper jaw is integrated, and the lower is not. This implies that the lower jaw in RD species is free to evolve a wide array of geometries, which may be advantageous for substrate feeding species, where demands on the lower jaw should be more variable relative to the upper jaw apparatus. However, this is not to say that there is a complete lack of integration between the upper and lower jaw, as modularity is a matter of degree rather than an all-or-nothing phenomenon [12] . Also, it is important to note that patterns of divergence among SD and RD are still acting within the overall context of a preorbital module (i.e., both upper and lower jaw for SD, upper jaw for RD,) suggesting that the rate and direction of phenotypic evolution is being dictated by historical constraints that are manifested in patterns of covariance and modularity. In other words, putative character displacement between SD and RD species in Malawi cichlids may be proceeding along genetic lines of least resistance [5, 53].

4.2. Origins for Adult Patterns of Modularity: Developmental Mechanisms. Although there are a number of possible functional explanations for patterns of craniofacial modularity, it is important to remember that selection must work within the context of developmental systems to improve functional performance. That is not to say development inherently constrains evolution, but rather that it can direct its outcome in concert with selection. In fact, simulations have shown that some degree of order may actually be required for evolution to proceed with ease [60]. It is, therefore, probable that the patterns of craniofacial modularity identified here, while probably causing an increased propensity for adaptations involving the oral jaws, are also dictated by underlying developmental processes. Clues to these potential processes may lie in early embryological events during the formation of craniofacial anatomy in fishes (see [61] for a similar view in mammals).

Structural progenitors of the ossified structures in the preorbital region of the skull include the trabeculae and ethmoid cartilages (i.e., anterior neurocranium), palatoquadrate (i.e., upper jaw precursor), and Meckel's cartilage (i.e., lower jaw precursor). All of these structures are derived from the same population of anterior cranial neural crest (CNC) cells that migrate away from neural tissue beginning at approximately 12 hours afterfertilization (hpf) in zebrafish [62]. Thus, the preorbital region of the skull is defined early in development, and these events may underlie the persistence of a preorbital module among African riftlake cichlids. For instance, LM cichlids show integration between the upper and lower jaws, suggesting that this developmental hypothesis may have particular merit for this adaptive radiation.

The modular divisions between the upper and lower jaws found between LV and LT may be influenced by slightly later developmental events. Fate mapping experiments in zebrafish show that at approximately 24 hpf the stomodeum forms as an invagination of the oral ectoderm, and both the pterygoid process and anterior neurocranium reside within a compact condensation of cells closely associated with dorsal edge of this structure, whereas Meckel's cartilage forms from cells ventral to this structure [62]. Thus, while early ontogenetic events (i.e., CNC migration) regionalize the skull along the anterior-posterior axis, slightly later events (i.e., formation of the mouth) are necessary to specify the dorsal-ventral identity of the jaws within the preorbital region of the skull.

Later still in development, the sequence of ossification in bones of the craniofacial region may play a role in determining patterns of modularity. Evidence from zebrafish and Nile tilapia (*Oreochromis niloticus*) show that the oral jaws (premaxillae, maxillae, and dentary) are among the first structures to become mineralized in the teleost head [63, 64]. In fact, the only other structures that are ossified as early as the oral jaws include the basio-occipital and opercle. Functional reasons have been attributed to this chronological pattern in teleost development [63, 65–67]. Specifically, bones involved in early basic functions such as respiration and feeding have been observed to ossify first. This suggests that the bones of the oral jaws and opercular regions of the skull are predisposed to reflect the patterns of variational modularity we have identified. Ossification sequence, and heterochronic shifts in this process, could, therefore, act as another early mechanism that sets the stage for craniofacial modularity throughout life history.

4.3. Origins for Adult Patterns of Modularity: Integrating Developmental and Functional Processes.

Beyond initial ossification, bone remodeling over ontogeny could represent another means of achieving modularity of the oral jaws and a way of simultaneously integrating developmental and functional mechanisms in a straightforward way. Bone is a dynamic, metabolically active tissue that is constantly being renewed and changed. Bone cells are strain sensitive and can transduce signals from mechanical loading into cues that result in either reduced bone loss or gain [68–71]. Disuse usually causes an acceleration of bone turnover, with resorption being the dominant process. Conversely, excessive strain can damage bone, which may in turn be repaired or further reinforced through remodeling. Importantly, both bone resorption and deposition involve highly conserved genetic and developmental pathways [72–74].

Mechanical stimuli may be particularly important for inducing adaptive patterns of modularity through the process of bone remodelling. Bone turnover tends to be most effective in areas of high stress, thus reducing the risk of injury [69]. In teleosts, the oral jaws are used for both respiration and food acquisition, but it is likely that the oral jaws are under the highest stress during food acquisition and processing, which should in turn provide the greatest stimulus for bone remodeling [72]. Indeed, several lab-based studies on cichlids have documented that different diet treatments can induce changes in bone and head shape [75, 76], demonstrating the ability of elements in the upper and lower jaws to respond to mechanical stimuli through changes in shape. Within the RD lineage of LM, it is certainly possible that a high degree of remodeling and plasticity of the lower jaw has led to a pattern of modularity, wherein the mandible lacks a measurable degree of integration across species. The lower jaw may be more amenable to remodeling due the greater degree of movement that it is afforded in the RD lineage. Alternatively, patterns of integration within the lower jaw may differ between species, resulting in a perceived lack of integration in the combined dataset. In either case, the conclusion that must be drawn is that the lower jaw is a highly evolvable trait within the RD lineage.

Perhaps the most compelling evidence for a fundamental link between developmental and functional processes comes from work in the BMP family of signaling proteins (reviewed by [70]). Critical roles for BMP signaling during bone and cartilage development are well established (reviewed by [77]), and variation in BMP expression over ontogeny has been associated with the origin and adaptation of key vertebrate innovations including the turtle shell [78], bat wing [79], cichlid mandible [44, 80], and bird bills [81–83]. All of these examples involve differential *Bmp* expression that is presumed to be due to mutational effects (either *cis* or *trans*), but several studies have also documented environmentally induced changes in BMP expression in skeletal tissue. Specifically, tensile stress has been shown to alter BMP expression during bone growth [84, 85], remodeling [86], and repair [87, 88]. Thus, a scenario wherein patterns of craniofacial modularity are established via early developmental mechanisms and then either reinforced or altered by functional processes might represent the true nature of variational modules within the cichlid skull. Examining how patterns of integration potentially shift over ontogeny and under different feeding regimes in different cichlid lineages would represent a fruitful line of future research.

4.4. Modularity and Evolvability of the Craniofacial Skeleton in Cichlids.

Recent reviews suggest that an extended evolutionary synthesis (EES) is necessary to account for the origins of variation that is acted upon by natural selection [6, 7]. The empirical center for the EES will lie in discovering the features of organisms that determine evolvability [7]. While specific definitions of evolvability are numerous and vary according to context, modularity figures prominently in these discussions insofar as it imposes a constraint on direction or speed of evolutionary change [12, 13]. In this context, we suggest that modularity can act as a "key innovation". While key innovations are typically defined by the appearance of an anatomical structure that precedes an adaptive radiation, as is the case for the pharyngeal jaws [26], we contend that patterns of modularity, whereby the cichlid oral jaws represent a module that allows them to change with a high degree of autonomy, have had a strong influence on the rate and direction of adaptive divergence in this group. This pattern of modularity is likely what has allowed for the rapid lengthening or shortening of the oral jaws relative to the rest of the head in cichlids and shape changes that comprise the major axes of variation in each of the three African rift lakes, and likely, it represents the template upon which additional changes in trophic morphology occur [28]. In other words, the evolution of this pattern of modularity may facilitate evolution, providing an example of the "evolution of evolvability" (see [7]). The degree to which these patterns are specific to cichlids, or represent a more generalized perciform innovation, will be an important area of future study. Several avenues may have lead to preorbital modularity; therefore, finding groups that lack this pattern of modularity and comparing rates of diversification will be important for identifying its potential role as a key innovation.

As discussed above, several avenues may have led to the consistent patterns of preorbital modularity we have discovered. In the order of their ontogenetic appearance, these include (1) migration and specification of progenitor cells, (2) dorsal-ventral division of the oral cavity, (3) sequence of ossification with early calcification of the jaws and operculum region, and (4) remodeling of bone in response to mechanical stimuli. These all represent separate hypotheses and processes that can be tested to understand the developmental and genetic basis of a preorbital module. We predict that each of these processes may play important roles in determining modularity in the cichlid head, depending on the lineage being queried. Fortunately, we have the means to assess patterns of modularity over ontogeny in cichlids and can statistically track when the patterns we have identified in adult cichlids begin to emerge. We also have the means to identify QTL associated with these anatomical modules and to track changes in gene expression during the emergence of these patterns [43, 44]. In all, cichlids represent an attractive model to reveal both the genetic basis of modularity and the evolvability of the craniofacial skeleton.

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Review Article

Mating and Parental Care in Lake Tanganyika's Cichlids

Kristina M. Sefc

Department of Zoology, Karl-Franzens University Graz, Universitätsplatz 2, 8010 Graz, Austria

Correspondence should be addressed to Kristina M. Sefc, kristina.sefc@uni-graz.at

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Cichlid fishes of Lake Tanganyika display a variety of mating and parental care behaviors, including polygamous and monogamous mouthbrooding and substrate breeding, cooperative breeding, as well as various alternative reproductive tactics such as sneaking and piracy. Moreover, reproductive behaviors sometimes vary within species both in space and in time. Here, I survey reports on mating and parenting behaviors of Lake Tanganyika cichlid species and address the evolution of mating and parental care patterns and sexual dimorphism. Notes on measures of sexual selection intensity and the difficulties of defining mating systems and estimating selection intensities at species level conclude the essay.

1. Introduction

Mating and parental care patterns are shaped by evolutionary mechanisms, and in turn, reproductive behavior bears on the course of evolution. Both directions of this feedback loop have received attention in theoretical and empirical studies (e.g., [1–3]), but a number of issues around the interplay of sexual selection, ecology, mating, parenting, and phenotypic divergence remains controversial (e.g., [1, 4, 5]). Hypotheses have been tested in taxa ranging from yeast to humans [6, 7], with a strong case made for fishes as models to study sexual selection and parental care by Amundsen [8]. The opportunities offered by fishes are manifold: different ecological backgrounds, a variety of life histories, including sex change and alternations of reproductive tactics as well as fixed behavioral polymorphisms, external and internal fertilization, brood care ranging from none to biparental and even cooperative, and different levels of sexual dimorphism and speciation rates, allow to address consequences of sexual selection as well as factors influencing it. Except for sex change (but see [9]) and internal fertilization, cichlid fishes provide a broad width of study topics at varying phylogenetic levels. In particular, the diversity in morphology, ecology, mating, and parenting behavior of the Lake Tanganyika (LT) cichlids matches their phylogenetic diversity [10], and their potential for comparative phylogenetic studies has been tapped in various contexts [2, 3, 11–18].

The comprehensive synopsis of parental care and mating systems of Lake Tanganyika's cichlids by Kuwamura [19] has become a widely used source of information to researchers in this field. Kuwamura classified the mating systems of LT cichlid species into monogamy, characterized by biparental care or consistent spawning with the same partner, harem polygyny with the male territories including those of several females, male-territory-visiting polygamy, in which females visit the males' territories for spawning but do not form pair bonds, and nonterritorial polygyny, in which males defend spawning sites only during courtship. All cichlids perform brood care, either by mouthbrooding or by guarding eggs and fry (substrate breeding), or, as in several species, by a mixture of both, when mouthbrooding of eggs and small fry is followed by guarding of free-swimming fry. Kuwamura [19, 20] further classified the parental care patterns of LT cichlids into biparental guarding in monogamous substrate breeders, maternal guarding in haremic substrate breeders, mouthbrooding (sometimes followed by guarding) in monogamous species, and maternal mouthbrooding in polygamous species. True to the title of his article [19], Kuwamura developed a model of the evolutionary transitions between parental care patterns in LT cichlids and discussed the forces and opportunities driving these changes. A list of species with parental care, nest site, and mating system type completes his compilation. Since the publication of Kuwamura's article in 1997, further field and laboratory work and, more recently, genetic parentage analyses have unearthed even more detail about the reproductive behavior of LT cichlids. In particular, attention to alternative reproductive tactics (especially cooperative breeding) has ever been increasing, and a small number of selected species became prominent subjects to address the causes, functions, and mechanisms of different behavioral options. The role of natural selection in the evolution of sexual size dimorphism in shell breeding cichlids has been established by a series of studies. Finally, recent molecular phylogeny-based approaches to the evolution of mating and parental care patterns provided detailed reconstructions of behavioral character transitions, with good support for Kuwamura's original model.

The present paper collects both pre- and post- 1997 data on LT cichlid mating and parenting behavior, then summarizes studies addressing the evolution of mating and parenting patterns in cichlids and of sexual dimorphism in certain LT cichlid species, and concludes with notes on the estimation of sexual selection intensity. Intraspecific variation in reproductive behavior, the employment of various alternative tactics, and finally behaviors, which cannot unambiguously be assigned to one of the defined mating system categories (e.g., the combination of lek and resource-defense mating system characteristics by *Simochromis pleurospilus* [21]), render the classification of species-level mating systems difficult, and no attempt is made here to update Kuwamura's categorization of species by mating or parenting behavior.

2. Substrate Breeding and Mouthbrooding

Substrate breeders (Lamprologini and Boulengerochromini, Table 1) attach their adhesive eggs to different solid surfaces meeting species-specific requirements in orientation, angle, and seclusion. Hatched embryos are often transferred in the female's mouth to rock crevices or pits [22–25]. Larvae of some species remain close to the substrate and spread horizontally while those of others expand their area into the water column [23]. Brood care includes egg cleaning and fanning, as well as defense from predators. The eggs and fry of mouthbrooders are incubated in the buccal cavity of one or both of their parents. Advantages of mouthbrooding include protection from predators and environmental stressors [26], the possibility to breed independently of the availability of suitable surfaces for egg attachment [27], and the possibility to move fry to suitable habitats for release [26], but also incurs considerable costs (see below). Egg and clutch sizes differ between substrate breeders and mouthbrooders. The eggs of the mouthbrooders are typically quite large with diameters up to 7 mm (except for some biparental mouthbrooders [28]), and clutch size is naturally restricted by the capacity of the buccal cavity to <50–80 eggs depending on egg size and brooder body size [28–31]. In contrast, eggs of substrate breeders are smaller with diameters around 2 mm [28], and clutch sizes vary widely between species, ranging from 10–20 eggs in the small shell breeders to hundreds and thousands of eggs in larger species [28, 32, 33]. Time to hatching is positively related to egg size [11] and takes three to five days after fertilization in substrate breeders [23, 32, 34, 35] and three to eight days postfertilization in mouthbrooders [36, 37]. The duration of posthatching brood care is often longer in substrate breeders than in mouthbrooders and varies widely among species of both groups (see below).

Table 1: Alphabetical list of genera mentioned in the text and their tribal association according to Koblmüller et al., [10]. Lamprologini and Boulengerochromini are substrate breeders, all the other LT cichlids are mouthbrooders.

Genus	Tribe
Altolamprologus	Lamprologini
Asprotilapia[2]	Ectodini
Aulonocranus	Ectodini
Boulengerochromis	Boulengerochromini
Chalinochromis	Lamprologini
Ctenochromis horei[1]	Tropheini
Cyathopharynx	Ectodini
Cyphotilapia	Cyphotilapiini
Cyprichromis	Cyprichromini
Eretmodus	Eretmodini
Gnathochromis pfefferi[1]	Tropheini
Haplotaxodon	Perissodini
Julidochromis	Lamprologini
Lamprologus[3]	Lamprologini
Lepidiolamprologus[3]	Lamprologini
Limnochromis	Limnochromini
Microdontochromis[2]	Ectodini
Neolamprologus[3]	Lamprologini
Ophthalmotilapia	Ectodini
Paracyprichromis	Cyprichromini
Perissodus	Perissodini
Petrochromis	Tropheini
Plecodus	Perissodini
Pseudosimochromis	Tropheini
Reganochromis	Limnochromini
Simochromis	Tropheini
Spathodus	Eretmodini
Tanganicodus	Eretmodini
Telmatochromis	Lamprologini
Triglachromis	Limnochromini
Tropheus	Tropheini
Variabilichromis[3]	Lamprologini
Xenotilapia	Ectodini

[1] The genus names *Ctenochromis* and *Gnathochromis* are shared with species belonging to other tribes [10, 38].

[2] within *Xenotilapia* [38].

[3] Current generic assignments of *Lamprologus*, *Neolamprologus*, *Lepidiolamprologus*, and *Variabilichromis* species may differ from those in earlier publications.

In all LT cichlid species, females invest in brood care, either with or without male assistance. Male investment in reproduction is not entirely restricted to direct care-taking and nest defense; sometimes, males also provide resources such as foraging opportunities to females prior to spawning and thereby supposedly increase female fertility and their own reproductive output. In several species with uniparental maternal brood care, this kind of male investment in reproduction has been observed. In a population of *Neolamprologus tetracanthus*, harem-holding males avoided feeding in the females' territories, probably in order to preserve food resources for females, to provide them with an incentive to stay, and to promote their gonadal maturation [39]. Likewise, no paternal brood care is provided in the mouthbrooding *Tropheus* species, as females mouthbrood their offspring by themselves. Prior to spawning, however, the female *Tropheus* (except for *T. duboisi*) stay in their mates' territories for several days to weeks, where they feed heavily under their mates' protection [40]. Upon spawning, females leave their mates to mouthbrood solitarily. Paired females in a population of *Tropheus* sp. "black" [41, 42] had higher gonadosomatic indices and condition factors than solitary females, which suggests that the resources available in the males' territory are crucial for gonadal recovery and maturation of ovaries [40]. Opportunities for efficient and protected feeding in the courting males' territories are also used by females of the maternal mouthbrooder *Simochromis pleurospilus* [21].

2.1. Mating and Parenting Behavior of Substrate Breeders. In substrate breeders, brood care is carried out by both parents or by the female alone or with the assistance of helpers, and resource distribution and brood care requirements often determine whether or not individuals—usually males—can have more than one mating partner at a time. Harem breeders with little, if any, paternal brood care include the lamprologines *Lepidiolamprologus profundicola*, *L. lemairii*, *Altolamprologus compressiceps*, *Neolamprologus furcifer*, *N. mondabu*, and *N. modestus* [19, 24, 32, 34, 43, 44]. In contrast, exclusive monogamy and biparental guarding were observed in *Neolamprologus tretocephalus* [24, 43, 44], *Boulengerochromis microlepis* [45], and *Variabilichromis moorii* [35, 46, 47]. Males of several predominantly monogamous lamprologine species (*Lepidiolamprologus elongatus*, *L. attenuatus*, and *Neolamprologus toae* [22, 32, 44, 48]) sometimes breed with two and occasionally more females when adjacent breeding sites are available [24]. Parental roles are often divided between the sexes, with the smaller partner, which is in many species the female, remaining closer to the fry and providing direct brood care and the larger partner defending the peripheral parts of the territory (or the adjacent breeding sites) against intruders [19, 35, 43, 46, 48–50]; but see [47]. In biparental *Telmatochromis temporalis* and *Neolamprologus toae*, removal of the nest-tending males resulted in the loss of the clutches [22, 51], which exemplifies the necessity of biparental care as a constraint to polygyny. Sexual conflict over care became apparent in a study of *Julidochromis ornatus*, where the larger fish of the pair, irrespective of sex, induced their smaller partners to spend more time close to the brood and provide direct brood care, and the difference in the amount of care between the sexes increased with increasing size differences [50]. Size-dependent division of labor was also observed in other *Julidochromis* species and contrasts with the situation in most other substrate breeders where direct brood care is usually provided by the female (D. Heg, pers. comm.).

The duration of brood care varies between a few weeks to several months. *Lepidiolamprologus profundicola*, *L. lemairii*, *Lamprologus callipterus*, *Telmatochromis vittatus,* and *Altolamprologus compressiceps* do not guard free-swimming young, and brood care extends to no more than 2-3 weeks [25, 52, 53]. Including guarding of free-swimming fry, parental care is performed for 4-5 weeks in *Neolamprologus furcifer* [34], for 8–12 weeks in *Lepidiolamprologus attenuatus*, *L. elongatus* [32], *Neolamprologus modestus*, and *N. toae* [23], for 14 weeks in *Variabilichromis moorii* [35], and for 20 weeks in *Neolamprologus tretocephalus* [32]. In *N. furcifer*, the next brood was often produced before the previous one had left the territory of their mother [34].

Years of genetic parentage studies in fish have demonstrated that the social behavior does not always reflect the true shares in parentage and have led to the distinction between social and genetic mating systems [54]. This applies to cichlids as well: an exceptionally high level of multiple paternity was found in broods of the socially monogamous *Variabilichromis moorii*, where each of ten broods was sired by two to >10 males. In contrast, all offspring in a nest shared the same mother. The nest-tending males of this species apparently suffer substantial cuckoldry and provide care for a large number of unrelated fry [55]. Alloparental brood care is also provided by males of the socially monogamous biparental substrate breeder *Telmatochromis temporalis*. In this species, females spawn in small holes or under stones and nest entrances are too narrow for the large, territorial males to enter. However, small males stayed inside the nests of some pairs and sired offspring in several instances [48]. These small males had high gonadosomatic indices, indicating that they represent a sneaker phenotype adapted to sperm competition with the paired male. Several of the nests with small males contained offspring sired by both territorial and sneaker males. Moreover, a small number of nests without small males contained offspring, which were unrelated to one or even both of the nest-tending individuals, perhaps as a consequence of replacement of the parental fish. Some of the paired males were also observed to visit one or two additional females [48]. In another study, large unpaired males were observed to attack nest owners, court with paired females, and then leave the nests again in the care of the nest owner [56]. This pirating behavior might be a source of unrelated offspring in nests without sneaker males.

2.2. Shell Breeding. A specialized form of substrate breeding in lamprologine cichlids is shell breeding, whereby the inside of empty gastropod shells is used as a protected nest site [57]. Several substrate breeders facultatively utilize shells in addition to rock crevices and holes (*Lamprologus lemairii*, *L. laparogramma*, *L. signatus*, *Lepidiolamprologus pleuromaculatus*, *Neolamprologus caudopunctatus*, *N. fasciatus*, *N.*

leleupi, *N. multifasciatus*, *N. similis*, *Telmatochromis dhonti*, and *T. vittatus*), while others are specialized, obligatory shell breeders (*Altolamprologus* sp. "shell" of Cameron and Nkamba Bay, *Lamprologus callipterus*, *L. meleagris*, *L. ocellatus*, *L. ornatipinnis*, *L. speciosus*, *Lepidiolamprologus boulengeri*, *L. hecqui*, *L. meeli*, *Neolamprologus brevis*, *N. calliurus*, and *Telmatochromis temporalis* "shell-bed dweller" [58, 59], summarized in [57, 60]). Shell size constrains the body size of the shell-breeding cichlids; in some species, both sexes are sufficiently small to fit into shells (e.g., *L. ocellatus*) while other species display extreme size polymorphism with small females and males too large to enter the shells (most notably *Lamprologus callipterus* [61, 62], see below). Males control one to several females, and harem formation and size may depend on shell availability (e.g., in *L. callipterus* [57, 63] and *T. vittatus* [64]). While beneficial to the male, the recruitment of additional female breeders may conflict with the interests of the resident female [65–67]. In the facultatively polygynous *Lamprologus ocellatus*, males control female access to shells. In aquarium experiments, aggression by a resident female delayed the settlement of additional females, and mutual aggression among females continued in established harems. Peace-keeping interventions by the males occurred in the majority of aggressive situations and were crucial for the maintenance of the harem [66]. Further experiments suggested that the fitness of harem females suffers from offspring mortality due to aggressive interactions between juveniles from different cohorts [67].

Maternal guarding predominates among the shell breeding species; only the facultative shell breeders *N. caudopunctatus* and *N. leleupi* are biparental guarders [19, 20, 57]. At a study site in southern Lake Tanganyika (Wonzye Point), a population of a strict shell breeder, which was initially identified as *Neolamprologus meeli* but later recognized as a new species *Lepidiolamprologus* sp. "meeli-boulengeri" [68], contained ten monogamous pairs and two harems with two and five females, respectively, and both males and females defended against nest predators [69]. Genetic parentage analyses of these monogamous and polygynous breeders and the young in their nests revealed that about one-third of the 19 young of monogamous pairs were not related to either the male or the female, and three additional young were not related to the male; furthermore, the two analyzed young of a polygynous group were not related to the harem holder. Sneaking and stepfathering were suspected to be the origin of unrelated young [69].

Alternative reproductive phenotypes and tactics, including cooperative breeding, have been observed in many more LT cichlid species and are addressed later in further detail.

2.3. Mouthbrooding. LT cichlids other than the Lamprologini and *Boulengerochromis microlepis* are mouthbrooders. Several combinations of mating and mouthbrooding behaviors exist. In some species, mouthbrooding of eggs and small fry is followed by biparental guarding of free-swimming fry, which are retrieved into the parents' mouths when danger is perceived. In most mouthbrooders, however, brood care is performed exclusively by the female, and periodical release of fry for feeding has been reported in only a few maternal mouthbrooders including *Ctenochromis horei*, *Simochromis pleurospilus,* and *Tropheus* spp. [20, 31, 70, 71].

The costs of mouthbrooding, such as reduced growth, delayed gonadal recovery, and increased intervals between spawning [37, 72–75], are particularly high when the breeding parents feed little or not at all during mouthbrooding [20, 31, 73, 76] and become even higher when parents expend energy and incur predation risks for buccal feeding of their young to subsidize their growth and prolong protective brooding past resorption of the yolk [31, 76]. Mouthbrooders of some species take up food to nourish both their fry and themselves. Brooding *Cyphotilapia frontosa* achieved 25% of the gut fullness index of nonbreeders but did not recover their gonads while they were breeding [74]. Buccal feeding started while young were still in an early developmental stage with large yolk sacs, and gut fullness of young increased over the long mouthbrooding period (54 days [30]) [74]. Continuous foraging during mouthbrooding was also observed in *Tropheus duboisi*. The gut fullness index of mouthbrooding females amounted to 80% of that of nonbreeders, and no difference in condition factors was detected between breeding and nonbreeding females [31, 77]. Likewise, brooding *Microdontochromis* (*Xenotilapia* [38]) *rotundiventralis* parents had only slightly lower food picking rates and the same condition factors as nonbreeding fish, and females even recovered their gonads during the breeding period [28]. Buccal feeding requires some extra room in the buccal cavity. The necessary reduction of clutch size may be compensated by shorter spawning intervals of females if they are able to remain in good condition by feeding themselves along with their fry [31].

The duration of continuous mouthbrooding is shorter in species with a subsequent guarding phase than in those species which mouthbrood their fry till independence. Mouthbrooding and guarding last for 1.5 and 4.5–7 weeks, respectively, in *Haplotaxodon microlepis* [78] and *Perissodus microlepis* [79], for 2 and 0.5–3.5 weeks, respectively, in *Xenotilapia flavipinnis* [80], for 2 weeks each in *Simochromis pleurospilus* [71], and for 2-3 and 0–4 weeks, respectively, in *Ctenochromis horei* [70]. Without subsequent guarding, 3-4 weeks of mouthbrooding are shared between females and males in *Tanganicodus irsacae* [37] and *Eretmodus cyanostictus* [75], and 4-5 week long maternal mouthbrooding is performed in *Tropheus* spp. [31]. Particularly long continuous mouthbrooding for 54 days (i.e., almost 8 weeks) was observed in *Cyphotilapia frontosa* [81].

The duration of mouthbrooding differs not only between species, but also sometimes between individuals. While there was little variance in the duration of mouthbrooding in *Tropheus moorii* (n = 7; 33.2 ± 1.1 days) [31], *Ctenochromis horei* females mouth bred for shorter but more variable periods (n = 8; 19.1 ± 3.1 days) [70] and were shown experimentally to prolong mouthbrooding when exposed to fry predators [82]. Tuning of reproductive investment may be even more comprehensive. In *Simochromis pleurospilus*, females adjust their reproductive rate and offspring size according to the environmental conditions, which they had encountered as juveniles. Females experimentally raised in poor conditions produced larger eggs and young and spawned at shorter intervals than females raised on a high-food diet, irrespective of the conditions during adulthood [71, 83]. *Simochromis pleurospilus* juveniles disperse into the shallow habitat, whereas the adults occupy deeper water. Therefore, the conditions experienced during the mother's own juvenile phase are a better predictor of her offspring's juvenile environment than the conditions in her current habitat [71]. The impact of egg size on potential survival benefits of young was pronounced when food was scarce. Young hatched from large eggs maintained a size advantage over young from small eggs, had higher burst swimming speed, and, importantly, spent less time foraging and more time sheltering, which—in a natural setting—should markedly reduce their predation risk. In contrast, young raised with plentiful food supplies behaved similarly regardless of egg size [84].

2.4. Mating Behavior and Paternity in Maternal Mouthbrooders. In maternal mouthbrooders, the investment in reproduction, in terms of energy and time, is clearly skewed towards the female. Mating generally occurs in the males' territories, which can be arranged in expanded leks, and polygamy is the norm in these species. Indeed, multiple paternity was demonstrated by genetic data in some of the broods of *Ctenochromis horei* with up to five sires per brood [85] and *Simochromis pleurospilus* with one or two sires per brood [86]. Multiple paternity can result from successful alternative male reproductive behavior such as sneaking or from deliberate polyandry when females visit several males for spawning [20]. The latter was observed, for example, in *Paracyprichromis brieni*, in which males establish courtship territories in the water column near rocks [87], in the lekking, crater-building *Cyathopharynx furcifer* [88, 89] and *Ophthalmotilapia ventralis* [90], and in *Ctenochromis horei*, where a female temporarily escaped the mate guarding dominant male [70]. A female *Pseudosimochromis curvifrons* was seen visiting and courting with two males, but no spawning occurred on this occasion [91]. Intrusions by neighboring territory owners and attempts of parasitic spawning may also cause a female to interrupt spawning with her current mate and perhaps later resume spawning with a different male, as observed in, for example, *Gnathochromis pfefferi* [92] and *Ophthalmotilapia ventralis* [93]. In contrast, females of another open-water spawner, *Cyprichromis microlepidotus*, were observed to spawn their entire clutch with one male [87], and several of the genetically analyzed broods of *Ctenochromis horei* (43%) [85] and *Simochromis pleurospilus* (65%) [86] were in fact sired by a single male each. Only one or two broods have so far been analyzed with respect to genetic relatedness in other polygamous maternal mouthbrooders (the Tropheini species *Simochromis diagramma*, *Petrochromis fasciolatus*, *P. orthognathus,* and *Gnathochromis pfefferi*). Except for one of the two investigated broods of *P. fasciolatus*, the Tropheini broods were sired by a single male each [94]. The peculiar mating system of *Tropheus* spp. (excluding *T. duboisi*) with pair bonding prior to spawning and maternal mouthbrooding has already been mentioned above. No evidence of multiple paternity was found in a total of 19 broods of *Tropheus moorii* from southern Lake Tanganyika [94], and although the males siring the broods were not identified, it is likely that they are indeed those with whom the females paired prior to spawning [40].

2.5. Biparental Mouthbrooding. In monogamous mouthbrooders with biparental care, we find maternal mouthbrooding followed by biparental guarding in *Perissodus microlepis* [79], *Plecodus straeleni* [20]), and *Xenotilapia papilio* [19]. Biparental mouthbrooding, whereby the female broods eggs and early fry before the male takes over all (or part, as in *Microdontochromis rotundiventralis* and *Haplotaxodon microlepis*) of the larger fry, is either followed by biparental guarding as in *Xenotilapia longispinis*, *X. flavipinnis*, *X. spilopterus*, *Asprotilapia* (*Xenotilapia* [38]) *leptura* [19, 80], and *Haplotaxodon microlepis* [78] or occurs without guarding as in *Xenotilapia boulengeri* [80], *Microdontochromis rotundiventralis* [28], *Eretmodus cyanostictus* [37, 95, 96], and *Tanganicodus irsacae* [37]. Pairs of *Eretmodus cyanostictus*, *Xenotilapia flavipinnis*, *X. spilopterus*, and *Asprotilapia leptura* remain together for several breeding cycles [28, 75, 80].

The remaining Perissodini species (in addition to the above-mentioned *P. microlepis*, *P. straeleni* and *H. microlepis*) are also biparental care givers, but it is not known whether it is maternal or biparental mouthbrooding that precedes biparental guarding [19]. In contrast to the biparental *E. cyanostictus* and *T. irsacae*, another Eretmodini species, *Spathodus marlieri*, performs exclusively maternal mouthbrooding [37], and no information exists on *S. erythrodon*. Three Limnochromini species, *Limnochromis auritus*, *Reganochromis calliurus,* and *Triglachromis otostigma*, were described as biparental mouthbrooders with guarding in the popular science literature [41]. Several Ectodini species in addition to the above-mentioned *Xenotilapia*, *Microdontochromis,* and *Asprotilapia* species are also biparental mouthbrooders, while other species of the tribe perform maternal brood care [97, 98].

Advantages of biparental over uniparental care during the guarding phase of monogamous mouthbrooders can readily be conceived with regard to defense against fry predators, whereas the adaptive value of monogamy is less clear in species lacking the guarding phase. One conceivable advantage is the doubling of brooding space resulting from the division of broods between the parents, as in *Microdontochromis rotundiventralis* [28], but different explanations must be found for species in which females shift the entire brood to males. The constraints on mate desertion have been discussed in some of the above-cited studies, and were addressed in more detail in *Eretmodus cyanostictus*. Genetic parentage analyses of broods collected in the field confirmed that the species is both socially and genetically monogamous [99]. Different reasons for monogamy were proposed: costs of desertion arising from remating prospects were identified by Morley and Balshine [95], who concluded from removal experiments in the field that monogamy results from the need to guarantee high-quality mates and territories in a competitive environment, and by Taylor et al. [99] who argued that the difficulty of finding a new partner, given a male-biased sex ratio and assuming costly locomotion (suggested by the reduction of the swim bladder) along with vigorous mate guarding preclude polygyny (see also [96]). Benefits of biparental mouthbrooding were identified by Grüter and Taborsky [100] after experimentally separating mouthbrooding females from their mates. After a prolonged incubation period, single mothers released smaller and less-developed young than paired controls, and it was concluded that monogamy was favored by the necessity of male care. In another experiment of Grüter and Taborsky [101], experimental manipulation of sex ratios did not significantly alter mate desertion rates, but sexual conflict about brood care became apparent as males took the offspring later when several females were present [101], and females made up for condition-dependent reduction of male care by prolonged incubation [102].

3. Mixed Broods: Farming Out and Fry Dispersal

Broods containing unrelated con- or heterospecific fry have been observed in both substrate breeders and mouthbrooders. In general, possible origins of foreign young include kidnapping, nest takeovers, egg stealing, accidental mixing among neighbors, fry dispersal, communal care, egg dumping, and farming out [103, 104]. With the exception of brood parasitism by the catfish *Synodontis multipunctatus* [105], brood mixing in the Lake Tanganyika cichlids has been attributed to farming out, nest takeover, and fry dispersal between schools. By farming out (also termed "young dumping" [104]), the parents transfer their fry to the nests or brooding sites of other breeders and abandon them to the foster parents' care. Benefits of delegating parental duties are quite obvious. The less intuitive adaptive value of brood adoption to the foster parents derives in some species from predation dilution and heightened success in mate acquisition [103, 104]. For mouthbrooders with their limited buccal brooding space, involuntary brood adoption may be disadvantageous, and targeted foster parents have been observed to drive away young-dumping adults [106, 107] and to be reluctant to accept foreign young [80, 108].

In the biparental mouthbrooder-guarder *Perissodus microlepis*, farming out was induced experimentally by removal of one parent during the guarding phase. Some of the remaining parents attempted—sometimes successfully—to put the brood under the care of another conspecific brooding pair, which was interpreted as adaptive behavior to cope with occasional mate desertion and increase the chances of brood survival [108]. Later, "farming out" of young was also observed under natural conditions [107] and as a tactic adopted by paired males, presumably to evade paternal investment [106]. Young *Perissodus microlepis* were also found in broods of other species (e.g., of *Lepidiolamprologus elongatus* [104]).

Brood mixing and farming out of young, both to conspecific and to heterospecific parents, occur in several species in addition to *Perissodus microlepis*. A number of observations of heterospecific brood mixing have been reported with guest species including several biparental mouthbrooders, *Cyprichromis* species, *L. elongatus,* and *L. attenuatus*, and hosts including *P. microlepis* and the substrate breeders *L. elongatus*, *L. attenuatus*, *L. profundicola*, *Neolamprologus caudopunctatus*, *N. pulcher*, *N. savoryi,* and *N. tetracanthus* [36, 109, 110]. Three out of 23 mouthbred broods of *Haplotaxodon microlepis* contained conspecific young of obviously different sizes, presumably as a consequence of farming out, and larger juveniles mixed with guarded broods of *P. microlepis* [78]. Free-swimming young of the biparental mouthbrooder *Microdontochromis rotundiventralis* were found under parental care of *P. microlepis*, *L. elongatus*, and several other substrate breeders [28]. One event of successful farming out of free-swimming fry and several unsuccessful attempts were observed in the biparental mouthbrooder *Xenotilapia flavipinnis*. Additionally, brood mixing was indicated by increasing numbers of young in several territories [80].

The mouthbrooders' habit of taking fry into the mouth facilitates the transport involved in farming out, but brood mixing may also be initiated by the young themselves. For example, large young *X. flavipinnis* invaded a neighboring territory and eventually were accepted by the guarding pair [80]. Likewise, foreign conspecific young in nests of the substrate breeder *Lepidiolamprologus* sp. "meeli-boulengeri" (*Neolamprologus meeli* in the original publication) were large enough to have entered their foster parents' territories on their own, although nest takeover could not be excluded as a source of nonkin young [69]. Similarly, replacement of breeders and immigration of large fry from neighboring nests were suggested as alternative explanations for mixed broods in *Julidochromis ornatus* [111]. Foreign fry were also detected among large mouthbred young of the maternal mouthbrooder *Simochromis pleurospilus* [86]. Mouthbrooding *S. pleurospilus* occasionally release late-stage fry to feed and then collect them back into their mouths, which may be an opportunity for neighboring females to accidentally or deliberately mix fry [20, 71].

4. Cooperative Breeding

Several of the substrate-breeding cichlids of LT breed in groups consisting of dominant breeders and a variable number of subordinate helpers. Heg and Bachar [112] listed 19–21 (counting aquarium observations of two species) cooperatively breeding LT cichlid species from several different lineages in the tribe Lamprologini [60, 113]. Characteristics of helper systems vary among LT cichlids, with differences in group sizes, degrees of helper-breeder relatedness, and levels of direct helper reproduction. Therefore, different hypotheses about benefits and costs of helping, such as kin selection, paying to stay, and helper reproduction as an alternative reproductive tactic, can be addressed and tested in the different species. *Neolamprologus pulcher* has been the prime model species among the LT cichlids to study causes, effects, costs, and benefits of helping and the regulation of group composition, group size, and division of labor [114–145].

Data on group composition and helping behavior of *Neolamprologus pulcher* have recently been reviewed by Wong and Balshine [119] and are summarized here only briefly. In fact, the first detailed studies of brood-care helping in fish were made in *N. brichardi* (conspecific with *N. pulcher* [146]) in northern LT [114, 147]. Subsequently, a wealth of field data were collected from *N. pulcher* in southern LT. Social groups are arranged in clusters and consist of dominant breeders and up to 20 male and female helpers, with an average group size of 7–9 individuals. Direct brood care, for example, fanning and cleaning of eggs and larvae, is usually provided by dominant females and female helpers [121, 130, 147]. Breeders and helpers of both sexes participate in territory defense and maintenance [147]. Both monogamy and polygyny occur, and although males holding several breeding territories were shown to be larger, less parasite-infected and to invest more in spermatogenesis, there was no difference in number and survival of young between monogamous and polygynous breeders [148]. Breeders are eventually replaced by dominant helpers [149], and the turnover rate is higher for male than for female breeders [150]. Offspring remain at home for a prolonged period of time, but eventually, some helper turnover results from dispersal into other groups, often associated with a rise in the helper dominance hierarchy [149, 151]. Female helpers are more likely than male helpers to inherit the breeding position of their relatives [150, 152], whereas dispersal rates are biased towards large males [153]. Probably as a consequence of both helper and breeder turnover, genetic relatedness of helpers to breeders declines with helper age, and helpers are more closely related to the female than to the male breeder [150]. Although related individuals may achieve breeding positions in the same group, apparently no measures are taken to avoid pairing among relatives [154].

Helpers were shown to reproduce in experimental groups (e.g., [121, 122, 141, 145, 155]). In field groups, genetic data confirmed a low level of female, but no male, helper reproduction [156, 157], but the detection of helper reproduction in the field may be compromised by the possible expulsion of reproducing helpers from the group [156]. Indeed, cage experiments in the field revealed that helper males do sometimes gain parentage (D. Heg, pers. comm.). Reproductive suppression of male helpers may make them inefficient sperm competitors to breeder males [133, 158]. The reproductive success of female helpers may be curbed by suppression of egg laying and the destruction of their clutches, although some reproduction may be tolerated in return for continued brood care [117, 130].

In Dierkes et al.'s study [156] of 12 social groups, maternity of the dominant females was confirmed in all but one group (in which the breeding pair had probably been replaced), whereas paternity of the dominant males varied from 0 to 100%. The fathers of the extra-pair young could not be identified. Paternity levels of harem males were not significantly different from those of monogamous males. Stiver et al. [157] examined five social groups from the same location and found both multiple maternity, in one case due to reproduction by a female subordinate, and multiple paternity, in two cases with offspring sired by dominant males from other groups. Paternity of dominant and subordinate males from neighboring groups was also observed in an experimental setup (D. Heg, pers. comm.).

A similar composition of cooperative breeding groups to that in *N. pulcher* was described in the closely related *Neolamprologus savoryi* [159]. Males were either monogamous or, when larger, polygynous with up to four females, each of which defended a separate subterritory (subgroup). Helper numbers ranged from three to 33, and mean group size was 14 individuals. Sexual size dimorphism between breeders was more pronounced in *N. savoryi* than in *N. pulcher*, entailing potential differences in life history strategies, for example, regarding age at reproduction and helper strategies, between the two species [159]. Genetic analyses of relatedness and parentage in two populations in southern LT (Kasakalawe and Kasenga) revealed a low level of helper reproduction, with higher reproductive success of male than of female helpers [160]. In groups with multiple breeding females, the females breeding within the same patch of stones were more closely related to each other than to breeder females from separated patches, which suggests that subgroups within a patch are founded by close relatives through expansion of the territory. Overall, subgroups and groups consisted mainly of related individuals with only 16–25% immigrants, and both patrilinear and matrilinear territory inheritance were common (33% and 52%, resp.). In contrast to *N. pulcher*, helpers in *N. savoryi* groups are more closely related to the dominant male than to the dominant female, probably as a result of a lower turnover rate of male breeders and a higher level of patrilinear territory inheritance [160].

The tiny, shell breeding *Neolamprologus multifasciatus* lives in groups of several reproductive males, females, and their offspring. Genetic analyses [161] identified these groups as extended families, with close relatedness among breeding males and between adult females and the alpha male. Mating occurred between full sibs and between offspring and parents, and large male helpers shared in reproduction. Exchange between groups is female-biased [65, 162]. Cooperative breeding is also performed in the closely related *N. similis* [112].

In all species of the genus *Julidochromis*, at least some of the breeding pairs are assisted by helpers. In a population of *J. marlieri* at Bemba, northern DR Congo, monogamous pairs predominated while a particularly large female bred with two males, and some of the breeders were assisted by one subordinate helper [49]. Similarly, Taborsky [104] observed helpers in several families of *J. marlieri* at Magara, Burundi. Following up the observed occurrence of polyandry, experiments demonstrated female-biased aggressiveness and dominance in this species [163].

Details of social interactions and genetic parentage have been studied in breeding groups of *J. ornatus* in southern LT [111, 112]. At Kasenga Point, Awata et al. [111] found that the majority of groups consisted of monogamous breeding pairs without helpers, followed by monogamous pairs with helpers, and a small proportion of groups representing polygynous (with two or three females) or polyandrous (with two or three males) harems with helpers. Typically, only one helper was present in a nest, but haremic nests could include up to six helpers. Male helpers were commoner than female helpers. In nearby Kasakalawe, Heg and Bachar [112] studied 28 groups consisting of breeding pairs and zero to five helpers. Importantly, the formation of several new groups could be documented, whereat breeding pairs established themselves at previously unoccupied sites and were joined by subordinates. In the Kasenga Point population [111], kinship analyses revealed that offspring in both monogamous and polygamous nests were either full sibs, half sibs, or unrelated, and the degree of intrabrood relatedness decreased with increasing size of young, consistent with both replacement of adults and immigration of unrelated young. Most of the helpers were not the offspring of the current breeders. The reproductive success of helpers was about as high as that of breeders, making helping an alternative option to pair breeding for small males [111]. Similar kinship structures and high rates of helper reproduction were detected in two additional populations of *J. ornatus* [164] as well as in *Chalinochromis brichardi* (Kohda et al.'s unpublished data cited in [111]), a close relative of *J. ornatus* [113]. In *J. ornatus*, the presence of sperm competition between male breeders and helpers was strongly indicated by the finding that both helpers and male breeders invest more in testis mass in cooperative breeding situations than do breeding males without helpers. Moreover, the gonadal investments of helpers and breeders of the same nests were positively correlated, and finally, offspring numbers increased with increasing testis mass [164]. Responses to perceived risk of sperm competition were also observed in the cooperative breeder *J. transcriptus*, where males kept in polyandrous groups developed larger testes than males in monogamous groups [165]. In the field, the breeding system of *J. transcriptus* resembles that of *J. ornatus* and *J. marlieri* with predominantly monogamous, biparental brood care and a smaller proportion of cooperative breeding groups. In experiments, the mating system depended on body size, with polygamous mating of large individuals of each sex and monogamous pairing among similar-sized individuals [166]. Female *J. transcriptus* prefer wedge-shaped rock crevices as nest sites, which apparently allow them to control paternity shares of differently sized males in polyandrous breeding groups and to reduce reproductive skew among males in a group: eggs laid on the wider part of the wedge are fertilized by large males, whereas males, which are small enough to enter the nest, escape aggression by large males and fertilize eggs in the inner part of the wedge [167].

5. Sneakers, Satellites, and Other Parasites

External fertilization, indeterminate growth, and high investment in brood care invite the employment of alternative reproductive tactics [168]. Individuals of many LT cichlid species exhibit a variety of phenotypes and behaviors to take their share in reproduction despite reduced investment in mate attraction and brood care. Helper reproduction in cooperatively breeding groups is one such example. Another example is the farming out of brood to conspecific pairs in order to reduce own brood care effort (note that interspecific brood care does not meet the definition of alternative tactics [169]). Different male phenotypes, associated with different reproductive behaviors, were detected in several substrate-breeding species, whereas reproductive parasitism in mouthbrooders was not found to be associated with pronounced morphological differentiation between bourgeois and parasitic individuals.

An extreme and well-studied example for divergence in male reproductive tactics is represented by the obligatory shell breeder *Lamprologus callipterus* [52, 61, 63], recently reviewed in [168]. Females enter shells completely, attach eggs to the wall of the shell, and perform brood care for about two weeks. The largest males, which are about 12 times heavier than the females [62], adopt the bourgeois tactic of courtship and nest defense, often preceded by active transport of scattered shells into their nests [52] and hold large harems with up to 18 females [57]. Other ways to come by or augment nests are shell stealing and territory takeover, typically associated with female expulsion and infanticide [52, 170]. Nest-holding males feed little or not at all, fuelling their "capital breeding" strategy on previously accumulated reserves, and extend their tenure to an average of 33 days [171]. Prior to becoming territorial, these males opportunistically spawn parasitically in the nest of bourgeois males by releasing sperm into the shell opening, but this behavior was assumed to result in only low reproductive outcomes [172]. In addition to these conditional tactics, there is also a fixed, genetically determined tactic of dwarf males [171], which are only 2.5% the size of nest males and considerably smaller than females. Dwarfs take advantage of temporary absences of the nest-holding males to wriggle past the females into the inner whorl of the shell, where they exploit the safety of their position to fertilize eggs in competition with the nest owners [52, 63, 171, 172]. The gonadosomatic index of dwarf males is much higher than that of nest-holding males [171]. Genetic analyses revealed that the dwarf males' share in paternity is low, which is perhaps compensated by their earlier onset of reproduction [63]. The polymorphism of reproductive pathways is probably maintained by frequency-dependent selection [63, 171]. Body size of dwarf males is determined by the small inner space of the shell left over by the breeding female and covaries with shell size and female body size among populations [173]. Apparently, the pronounced intrasexual size dimorphism among *L. callipterus* males is promoted by disruptive selection for large size in nest-holding males and small size in males adopting the parasitic tactic [173].

A similar variety of male tactics was observed in the hole-breeding *Telmatochromis temporalis*, which features large nest-holding males, small-sized males with large testes spawning inside nests and large pirates temporarily replacing the nest owner [48, 56] (see above). Small males with high gonadosomatic indices were also found in the shell-breeding *Neolamprologus brevis* (M. Aibara, pers. comm. to Katoh et al. [48]).

Four reproductive tactics were observed in a population of the facultative shell breeder *Telmatochromis vittatus* in southern LT, where this species breeds as guest in the large shell beds assembled by *L. callipterus* [174]. In this species, pronounced sexual size dimorphism allows the females to enter rock crevices or shells for spawning and brood care, while males in the upper size range spawn at the nest entrance. Large males defended shells and controlled several females; males even larger than the territorial males acted as pirates, temporarily ousted the nest owners during spawning and performed the posture of sperm ejaculation at the shell entrance; medium-sized males pair-spawned in single shells; small males acted as sneakers by spawning either inside the shell or at the shell entrance during absence of the nest-holding male. Sperm competition among territorial and parasitic males was inferred from the increasing gonadosomatic indices between pair spawners, polygynous nest holders, nest pirates, and sneakers [174], from similar differences in sperm swimming speed [175], and from the plastic increase in sperm longevity of territorial males when faced with the risk of reproductive parasitism [176]. Although the number of sneakers per nest is high, pirates present an even greater threat to nest holders as they procure unrestricted access to fertilization [64]. The home ranges of pirates include several nests, between which they travel repeatedly in order to detect ongoing spawning events. The incidence of piracy is high with 0.3 to 1.8 invasions per hour during the 3-hour period of spawning by the territorial male and is negatively correlated with the distance between nests and with the size of the nest owner [53, 64]. The quality of a nest is, therefore, determined not only by the number of shells it contains, which is positively correlated with the number of females at the nest [64], but also by its position relative to other nests, which predicts the risk of piracy. In fact, keeping the risk of piracy low seems to be a more important criterion for nest choice than nest size [53].

Parasitic reproduction was inferred from genetic parentage data in the biparental substrate-breeding cichlids *Lepidiolamprologus* sp. "meeli-boulengeri" (*Neolamprologus meeli* in the original publication [69]) and *Variabilichromis moorii* [55] (see above), but no alternative reproductive phenotypes and behaviors were observed, and the social status of the extra-pair males remains unknown. Spawning synchrony in *V. moorii* may constrain paired males to their own nests during the period when fertilizable eggs are available, such that extra-pair fertilization may mostly be achieved by unpaired males [55].

Extra-pair spawning and fertilization were also detected in several maternal mouthbrooding species. Some males parasitize on the mate attraction success of territorial, courting, colorful, or otherwise attractive males. *Paracyprichromis brieni* males establish courtship territories in the water column near vertical rock surfaces and lead approaching females to the rock surface for spawning [87]. Attempts of sneaking were observed in this species but not in another open-water spawner (*Cyprichromis microlepidotus*) mating away from the substrate, probably because sneaking males find hiding space and refuge more easily in the rocky area [87]. Interruptions of pair-spawning by neighboring or other conspecific males were also observed in *Gnathochromis pfefferi* [92], *Ctenochromis horei* [70], and *Pseudosimochromis curvifrons* [91]. In *G. pfefferi*, intruders were repulsed by the mating males, but in *C. horei*, the intruder quivered in parallel with the dominant male while the female nuzzled the dominant male's genital area, and extra-pair fertilization may have occurred. In a different population of *C. horei*, genetic analyses revealed multiple paternity of some broods, which could be due to both sneaking and deliberate polyandry [85].

In *P. curvifrons*, intruders either placed themselves in a spawning position parallel to the resident male or assumed a spawning position while the resident male and female were changing their display positions, which sometimes led to the female nuzzling the intruder's genital area. Intrusions occurred one to several minutes after the last spawning of the pair while the pair still continued reciprocal display and nuzzling, and sneak fertilization may depend on the extent to which eggs have previously been fertilized by the resident male. Intruders were either of the same size as or smaller than the resident males and seemed to be nonterritorial floaters [91].

In two closely related species, *Ophthalmotilapia ventralis* and *Cyathopharynx furcifer* [97], intruding males take advantage of the resident male's temporary distraction from defense. Territorial *O. ventralis* males spit sand onto horizontal rock surfaces to mark their mating territory by a small and rather untidy crater, to which they lead females for spawning. Quite frequently, floater males were observed to sneak into the territory and court the female, when the bourgeois male was temporarily absent chasing off other males. The small body size and good body condition of floaters suggested that floating (and sneaking) represented a transitional stage on the way to territoriality although males might also switch back and forth between the two behaviors [93].

The large mating craters built by males of *Cyathopharynx furcifer* (and an undescribed congener) and the activity associated with their construction apparently provide cues for female mate choice [89, 177]. The female deposits eggs in the crater and picks them up into her mouth after the male has passed over (and probably fertilized) them [88]. In a population in southern LT, the efforts of the crater-holding males were exploited by floaters, which occasionally succeeded to enter the crater, when the owner was occupied fending off other potential intruders, and rapidly performed spawning motions on the same spot where the crater owner had previously done so. Probably the intruding male mixed his sperm with that of the territory owner for the female to subsequently take up into her buccal cavity [178]. Four alternative male behaviors (sneakers, satellites, pirates, and female mimics) in addition to territoriality were described in one population of another crater-building species, *Aulonocranus dewindtii* [179].

Among biparental mouthbrooders, intraspecific brood farming out (see above) remains the only alternative reproductive tactic described so far.

6. Intraspecific Variation in Mating and Breeding Behavior

Interindividual variation in the number of mates, that is, monogamy versus polygamy, was detected in many of the studied populations, both in substrate breeders and in mouthbrooders (mentioned previously). A second component of mating system variability, which has repeatedly been encountered, is introduced by differences between populations, often correlated with differences in nest-site availability or predation pressure. In the substrate-breeding *Neolamprologus tetracanthus*, monogamy with male participation in brood care predominated in northern LT (Nyanza Lac), whereas males in southern LT (Wonzye) held harems with up to 14 females, and brood care was performed by females. Densities of *N. tetracanthus* were similar in the two locations, but nest predation pressure was higher in the northern population, such that biparental brood care may be indispensable in this population. Moreover, the lower density of potential nest sites in the northern location might also contribute to monogamy [180]. A relationship between nest-site (gastropod shell) density and mating system was also hypothesized in connection with disparate levels of polygyny (predominant monogamy versus predominant polygyny) detected in two studies of *Neolamprologus meeli* (now *Lepidiolamprologus* sp. "meeli-boulengeri" [68]) in southern LT [57, 69].

Differences in shell supply and shell sizes between localities may also influence the level of polygyny in *Lamprologus callipterus*, perhaps indirectly through an effect on male size [52, 57, 63]. In one location in southern LT (Wonzye), large shells are abundant and males grow to large sizes in order to be able to transport these shells to their nests; here, males hold large harems (average of 4 and maximum of 18 females). In a site in northern LT (Kalundu), large shells are less abundant, males grow on average somewhat smaller than in Wonzye, and maximum, but not average, harem size is smaller (average of 5.5 and maximum of 14 females). Finally, on the north-east coast (Rumonge), males do not transport shells, which already occur at high density in the habitat, and remain distinctly smaller and hold smaller harems (average of 2.4 and maximum of 7 females) (see also [181]).

Local shell size was proposed to determine not only the nest-holding male sizes but also the occurrence of dwarf males, which would only evolve in the presence of sufficiently large shells and when there was a lot to be gained by early onset of parasitic reproduction [63]. However, the more recent finding that dwarfs adjust their growth to available shell size [173] and observations of dwarfs at all of the three ecologically distinct locations (K. Ota, pers. comm.) reject this hypothesis for *L. callipterus*. It may still apply to another species, *Telmatochromis temporalis*, where a relationship was observed between the nest hole size and the occurrence of dwarf males [48]. In southern LT (Nkumbula (=Mbita) Island), nest burrows were too small for large males to enter, and small males with high gonadosomatic indices entered the nests to spawn inside. In contrast, in a population in northern LT (Bemba), the rocky crevices used for nesting can be entered by the nest males, and sneaker phenotypes were not observed there [48].

Geographic variation in social structure and in the occurrence of alternative reproductive phenotypes was also found in maternal mouthbrooders such as the crater-building *Aulonocranus dewindtii*, where nonterritorial males in one isolated population exhibited various alternative reproductive phenotypes not found in other places [179]. It was suggested that the limited opportunities for crater construction at this particular location imposed pressure on males to employ alternative reproductive tactics. In the likewise crater-building *Cyathopharynx furcifer*, the reported interpopulation differences in the mating and social behavior included the habitat type, on which territories and mating craters were established, the size of males defending mating craters, and the occurrence of parasitic reproduction. Craters were built both on rock and sand in Luhanga in northern LT but only on rock in Wonzye, southern LT. In Luhanga, small males held craters on sand, whereas in Wonzye, small males held territories without craters. Parasitic reproduction occurred only at Wonzye. Differences in predation pressure at the two sites were considered to account for these behavioral differences [178], but another, taxonomic rather than ecological, explanation is possible. A closely related, not yet formally described species (currently known as *C. foae*) occurs sympatrically with *C. furcifer* in southern LT while it is not clear whether both species or only *C. foae* occur in northern LT [33]. It is also not clear whether previous studies distinguished between the two species and putative intraspecific variation could in fact be an interspecific difference between *C. furcifer* and *C. foae*. The same applies to observed differences in female mate choice behavior between *Cyathopharynx* populations in northern and southern LT. Here, a relationship between female preferences and characteristics of the males' mating craters was detected in a southern (Kasakalawe) population of *Cyathopharynx furcifer*, but not in a northern (Bemba) population [88, 89]. Rather, in the latter population, the number of visiting females was related to the length and symmetry of the males' pelvic fins [88].

In *Ctenochromis horei*, dominant males monopolized mating in a northern population (Bemba) [70], whereas genetic parentage reconstructions in a southern population (Kalambo) were not compatible with monopolized reproduction but rather suggested that a large number of males sired offspring [85]. Moreover, there was a difference in the levels of multiple paternity of broods between two seasons, since all broods had two to five sires in March, while all but one October broods had only a single (but different) sire each. The sampling times fell into the rainy season (March) and the dry season, such that a seasonal effect is possible and requires further studies [85].

7. Evolution of Mating Systems and Parental Care Patterns

Models of the evolution of parental care patterns in fish have been summarized, for example, by Mank and Avise [182] and Balshine-Earn and Earn [183]. The stepping stone model assumes stepwise transitions starting from the absence of parental care followed by male-only care arising as a side effect of territoriality and defense of a spawning site, followed by a switch to biparental care in conditions either when care by both parents is required to assure survival of the brood or when females spawn only once in a season. Finally, changes in the operational sex ratio may increase the remating opportunities for one sex and promote mate desertion and uniparental (usually maternal) brood care. A competing model postulates independent origins of paternal, maternal, and biparental care from the ancestral absence of care. Comparative phylogenetic analyses across actinopterygian families accumulated stronger support for the independent origins than for the stepping stone model [184]. Within Cichlidae, however, maternal care (and paternal care in species of one genus, *Sarotherodon*) apparently evolved from biparental care systems, as predicted by the stepping stone model: the ancestral care type is biparental substrate-breeding, and several transitions occurred towards mouthbrooding and towards female-only care [2, 19, 183, 185, 186]. Additionally, among the Ectodini of LT, reversals from maternal to biparental mouthbrooding occurred in the genera *Xenotilapia* and *Microdontochromis* [97, 98].

The habit of mouthbrooding may have its origin in the oral transport of young by substrate guarders after hatching or repeatedly during the entire care period as, for example, in the LT cichlid *Boulengerochromis microlepis* [25]. Furthermore, biparental mouthbrooding, followed by biparental guarding in some LT cichlids, likely represents an intermediate form along the way from biparental substrate-breeding to uniparental mouthbrooding [19, 183]. Constraints on mate desertion in a biparental mouthbrooder, *Eretmodus cyanostictus*, were examined and discussed in several of the studies addressed above [95, 96, 99–101]. Uniparental, that is, female-only substrate-guarding evolved in biparental guarders as a response to opportunities for resource defense polygyny and harem formation, presented by high nest-site densities and numbers of receptive females [19, 24] and is contingent on the efficacy of uniparental guarding [22]. An additional constraint on both the social mating behavior and the employment of alternative tactics is imposed by lunar synchronization of spawning [55, 187–190], adopted by several substrate-guarding species presumably in order to increase nest-guarding efficiency and the safety of juvenile dispersal in moonlit and dark nights, respectively [24, 35], and by at least one mouthbrooder (*Cyprichromis leptosoma*) with less obvious adaptive value [36]. The observation of synchronized brooding in *Eretmodus cyanostictus*, which could have served to reduce remating opportunities and hence prevent male mate desertion in the biparental brooder [96], could not be confirmed in subsequent studies [75].

By assigning LT cichlids to two levels of sexual selection and tracing the correlated evolution of sexual selection intensity and parental care patterns on a phylogenetic tree, Gonzalez-Voyer et al. [2] concluded that transitions to female-only care were contingent on a previous intensification of sexual selection. As such, the study lends support to a view on the relationship between parental care and sexual selection [4] opposite to the more traditional notion that sex-specific differences in parental care investment underlie the operation and strength of sexual selection [191].

7.1. Mapping Evolutionary Transitions on Phylogenetic Trees: Not All Is Yet Resolved.

The great diversity of traits and the generally good resolution of gene trees make LT cichlids profitable and promising subjects for phylogenetic studies of trait evolution, not only with respect to parental care behavior, but also regarding life history and morphological traits [2, 12, 13, 15]. Nonetheless, when counting character transitions on phylogenetic trees of LT cichlids, it must be kept in mind that the placement of some taxa in mitochondrial gene trees may differ from their true relationships, for example, due to ancient incomplete lineage sorting and ancient hybridization among species, and that some nodes are endorsed only by low-statistical support [60, 68, 113, 192–194]. Taxon clades reconstructed from multiple nuclear loci (AFLP), at odds with mitochondrial clades in several instances, are often considered better representatives of species relationships than are mitochondrial gene lineages (e.g., [42, 194–196]), but often suffer from even lower statistical support for branching order than the mitochondrial trees (which can be improved by assembling data from a very large number of loci). A successful example of trait reconstruction on a well-resolved AFLP phylogeny is the study of parental care pattern evolution in the tribe Ectodini [98]. Beyond that, a useful basis for future studies could be established by building multilocus nuclear phylogenies, perhaps utilizing next-generation sequencing techniques rather than AFLP, of all LT cichlid species and tribes, and their allies outside LT. Despite some caveats, though, the general patterns identified so far are most likely bound to hold.

8. Sexual Dimorphism

The general correlation between mating systems and sexual selection intensity is reflected by the degrees of sexual dimorphism in taxa assigned to different mating systems [54], also when comparing the pronounced dimorphism in many of the female-only mouthbrooding LT cichlid species with the generally low levels of dimorphism in the biparental substrate breeders. Based on these apparent relationships, mating patterns and the occurrence of size and color dimorphism have been used to infer the action of sexual selection and approximate its strength [2, 12, 14, 15]. In a few cichlids of LT, however, presence or absence of sexual dimorphism is at least in part due to reasons other than sexual selection.

In the extremely size-dimorphic *Lamprologus callipterus*, the females, which breed in gastropod shells, can be less than 10 percent of the weight and half the length of the nest males, which fertilize clutches by spawning at the shell opening. While female size is constrained by the requirement to fit into shells, nest males must be large enough to be able to carry shells to their nests [52]. Studying a population in southern LT (Wonzye), Schütz and Taborsky [62] concluded from the observation of female preferences for large shells and the absence of sheltering within shells that *L. callipterus* descended from large- or intermediate-sized ancestors. This proposition is consistent with current phylogenetic data [60]. Hence, it was suggested that small female body size was a derived trait, following from natural selection imposed on females by the dimensions of their breeding substrate [62]. This was endorsed by subsequent field and laboratory studies [197] as well as modeling studies [198] addressing the selective forces acting on male and female body sizes. The absence of female preferences for male or nest size (number of shells) argued against intersexual selection for large male size, and small effects of intrasexual selection due to longer nest tenure of larger males were considered possible. A minimum size proved to be necessary for males to carry shells, whereas female body size was obviously limited by shell size and females adjusted growth to available shell sizes [197]. Likewise, the model suggested an effect of intrasexual selection and shell carrying on male size and a strong constraint on female size by the breeding substrate [198]. Contrasting with the above mate choice behavior, another study revealed female preferences for large males at a location, where shells were less abundant (Kasakalawe) than in the locality of the above study (Wonzye). When shells are scarce, females associated with small males face the risk of nest takeover or shell stealing by larger males and hence, the risk of expulsion and infanticide [170]. Furthermore, another population (Rumonge), which is notable for its small males, was recently reported to deviate from the general habit of using shells for breeding but not for sheltering. The habitat at this location provides no other refuge than shells, and at the same time, males at this location are not required to transport shells due to their natural distribution. Small male size in this population was suggested to be an adaptation to sheltering in shells made possible by the relief from shell transport [181]. In the populations other than Rumonge [181], both male and female body sizes were positively correlated with shell size, and the observed negative size allometry (the average size differences between males and females decreased with increasing average body size across populations) was explained by the female growth adjustment to shell size availability. Additionally, male-male competition for nests and fecundity selection on females were shown to affect body sizes [181]. All in all, the extreme sexual size dimorphism in *L. callipterus* appears to be a consequence of multiple and variable selection regimes, with only an auxiliary role for sexual selection.

In another shell breeder, *Lamprologus ocellatus*, sexual selection on females was shown to work against the rather moderate size dimorphism (4–6-cm versus 3–5-cm body size in males and females, resp.), since both male choice and female competition for breeding opportunities favor larger females [67]. Female body size is of course constrained by shell size, such that natural selection contributes to maintaining the existing dimorphism.

A maternal mouthbrooding species, *Tropheus moorii*, is notable for being sexually monochromatic while at the same time displaying most pronounced geographic color pattern diversity. Here, the diversification of male and female coloration was probably synchronized by the social system. Both sexes defend their feeding territories against competitors and neighbors of the same and opposite sexes. Body coloration is used as a means of communication in social interactions: Sturmbauer and Dallinger [199] distinguish between neutral, aggressive, subordinate and moving coloration (see also [200, 201]). Territoriality of males and females has been suggested to drive the evolution of conspicuous coloration while simultaneously constraining the evolution of sexual dichromatism [40, 199, 200, 202], but studies directly addressing this hypothesis are still needed. At any rate, the action of sexual selection in *Tropheus* cannot be excluded despite the absence of sexual dimorphism. For males, the possession of a sizeable territory is a prerequisite for mating success [40]. The outcome of male-male competition for good-sized territories, therefore, influences male reproduction, resulting in intrasexual selection (while the dual function as mating and feeding territory puts its defense under natural selection as well). Despite the pair-bonding prior to spawning, the operational sex ratio is likely to be male-biased due to the long female time-out periods during mouthbrooding and recovery [31, 203]. Concordant female preferences for certain males and concordant shunning of others were observed in laboratory female-choice experiments [204], such that intersexual selection may occur as well. In the natural setting, the variance in male reproductive success may be substantial, and sexual selection may be acting in *Tropheus* but has not been expressed as sexual dichromatism due to constraints by other forces.

9. Quantifying Sexual Selection

Estimates of sexual selection intensity in LT cichlid species have been derived from their mating systems [14] in combination with sexual dichromatism and size dimorphism [2, 15] for comparative phylogenetic studies. A cross-season comparison of potential sexual selection in a single population, based on genetic paternity data, employed indices representing the opportunity for selection [85]. Further proxies and measures of sexual selection include the operational sex ratio, potential reproductive rates of each sex, selection differentials, and Bateman and selection gradients, each with its own strengths and weaknesses [205]. Bateman's gradients and indices of the opportunity for selection, I, address some of the preconditions for the action of sexual selection, such as covariance between reproductive and mating success and variance in fitness among individuals of a population. While these conditions are prerequisite to sexual selection, positive values of their measures fail to tell whether or not sexual selection is indeed acting in the system [205]. For example, the opportunity for sexual selection is calculated as the variance in relative mating or reproductive success among individuals of a sex. However, any variance among males could also be random, in particular when there are fewer receptive females than males such that some males remain without a mate even though there may be no discrimination on the part of the females. The confusion of random and selection-based variance is acknowledged by the interpretation of I as the upper limit of sexual selection intensity [205–207]. Measures of I will become more meaningful with regard to the operation of sexual selection if they are combined with a demonstration of female choice or if they are calculated across several breeding seasons as random variances within seasons are likely to cancel out, at least to some degree, across seasons.

Few studies addressed mate choice and variance in reproductive success in LT cichlids. In *Cyathopharynx furcifer*, males differ considerably in their attractiveness to mate-searching females, and both their ornamented fins and their craters are candidate targets for sexual selection [88, 89]. Reproductive variance among males of *Lamprologus callipterus* results from complex interplays between intra- and intersexual as well as natural selection; both body size and nest characters may serve as mate-choice cues under different circumstances [170, 197]. In *Tropheus moorii*, female mate-choice may cause variance in male reproductive success, but the responsible traits have yet to be identified [203, 204, 208–210]. Not always, however, do mate preferences translate into actual mating patterns: size-assortative pairing of the biparental mouthbrooder *Eretmodus cyanostictus* [95] is not driven by preference (which would favor larger individuals) but rather by ecological constraints and intrasexual competition [211].

Another issue is the distinction among intrasexual, intersexual, and natural selection. Components of the variance in reproductive success often include territoriality and fertility, which may be considered to fall into the realm of intrasexual and natural selection, whereas being chosen for mating is clearly a matter of intersexual selection. Here, the researcher must take decisions on which individuals to include in the measurement of variance (All? Only nest holders? Only mated individuals? See [212] for the different implications of these approaches) and on the currency of fitness (Number of mates? Number of offspring? See [207]). While counting offspring rather than mates includes fertility (a component of natural selection), a restriction to mate numbers ignores unobserved alternative tactics and sperm competition, which can be highly relevant to the intensity of sexual selection [90, 213–216]. Collecting genetic data for the analysis of reproductive variance can be a challenge in itself [217]. In highly abundant species, such as *Tropheus*, *Variabilichromis moorii,* and other littoral LT cichlids, the probability of capturing close relatives is small even in large population samples due to the sheer numbers of individuals in a population [55, 203, 218], which makes the identification of nonparenting sires and dams difficult. In less abundant or pelagic species, dispersal may essentially create the same problem. Moreover, the number of nonreproductive individuals is difficult to establish. Some shortcuts are possible: for example, Wade and Shuster [219] showed that in haremic systems, the opportunity for sexual selection on males is proportional to the mean harem size of mated males, which can more easily be determined. For LT cichlids, this would allow to grade sexual selection intensity in polygynous populations with different harem sizes, provided that parasitic reproduction is negligible.

Selection gradients and selection differentials are more directly linked to sexual selection as they address the effect of the phenotype on reproductive success and the effect of sexual selection on phenotype evolution (contingent on heritability), respectively [205]. Obviously, though, it is necessary to identify the crucial trait(s) in order to include them into the analysis, and these may not be immediately obvious in some species (e.g., [204]).

Certainly, the best (or most feasible) approach to the assessment of sexual selection intensity depends on the aim and the scope of the investigation. Questions associated with the action of sexual selection at the population level are typically quite concise, for example concerning particular phenotypic traits and mate choice, and can be targeted by determining mate preferences, selection gradients, and selection differentials. At species levels, for example concerning correlates of species richness and phylogenetic studies of trait evolution, traits under selection will vary between taxa, and the available options will be limited to assessing the potential for sexual selection in the different taxa from benefits of multiple mating and from variances in reproductive success, to some degree represented by the mating system, or to infer the action of sexual selection from the presence of sexual dimorphism. However, alternative reproductive behaviors affect the distribution of reproductive success and hence sexual selection in different ways depending on the underlying social mating system and the details on status and success of the parasitic spawners [213–215]; moreover, sexual dimorphism may also result from natural selection (see above). Adding geographic and seasonal variations, it may in fact be very difficult to grade sexual selection intensity at the level of species.

10. Conclusions

Over the last decades, a tremendous amount of information on the mating and parental care behavior of LT cichlids has been assembled through extensive field work and, especially more recently, through experiments addressing the proximate and ultimate causes of different behaviors. Additionally, genetic techniques have proven valuable tools to complement observational data and, specifically, to target relatedness, parentage, and reproductive success.

Comprehensive insight into a range of topics associated with reproductive behavior and evolution has been gained by detailed studies of individual LT cichlid species, including cooperative breeding systems in *Neolamprologus pulcher*, *Julidochromis* spp., and *N. savoryi*, alternative male phenotypes and sexual size dimorphism in *Lamprologus callipterus*, sperm competition in *Julidochromis* spp., *L. callipterus,* and *Telmatochromis vittatus*, biparental brood care in *Eretmodus cyanostictus*, brood farming out in *Perissodus microlepis,* and context-dependent maternal investment in *Simochromis pleurospilus*.

Overall, alternative reproductive behaviors and phenotypes are well understood in some species, while for others, the evidence is presently rather anecdotal. Furthermore, in some species, alternative tactics have been described, but their success remains unknown, while in others, genetic parentage analyses indicated brood mixing and multiple mating, but information on the involved individuals is lacking. While each of the two approaches (genetic and behavioral) have provided valuable information, our understanding of mating and parental behavior will further benefit from an increased integration of behavioral studies and genetic analyses of individual reproductive success, a strategy which has already been practiced in several exemplary studies. Sexual conflict appeared in connection with harem formation and parental investment in at least four species (*L. ocellatus*, *N. multifasciatus*, *J. ornatus,* and *E. cyanostictus*), and may arise in many more species as well as on additional occasions such as the choice of mating and fertilization partners. The role of ecology in shaping behavioral patterns of LT cichlids has long been recognized and certainly merits further experimental work. Mate choice has been addressed in only few species, perhaps because sexual selection has long been considered of secondary importance in the evolution of LT cichlids. Nonetheless, a variety of potential mate-choice cues, including body coloration, body size, territory quality, nest, and mating crater characteristics, invite further studies about male and female decision making.

When the information on mating or parental care behavior originates from single populations and single points in time, as is still the case for many species, geographic and temporal variation may go unnoticed. Already, numerous examples document that intraspecific variation and alternative behaviors vitiate species-level classifications of mating and parental care systems, on the one hand creating a predicament for studies relying on the categorization of a species' behavior into one of few discrete categories and on the adherence to a certain behavioral pattern across short terms as well as evolutionary timescales. On the other hand, it is exactly this variability that provides the opportunities to examine how the interactions of different factors—such as genetic influences, sexual conflict, and all aspects of ecology—culminate in the display of certain behaviors by particular individuals or populations.

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Review Article

Male-Male Competition as a Force in Evolutionary Diversification: Evidence in Haplochromine Cichlid Fish

Peter D. Dijkstra[1, 2] **and Ton G. G. Groothuis**[1]

[1] *Behavioural Biology Research Group, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands*
[2] *Section of Integrative Biology, The University of Texas at Austin, 1 University Station-C0930, Austin, TX 78712, USA*

Correspondence should be addressed to Peter D. Dijkstra, pddijkstra@gmail.com

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It has been suggested that intrasexual competition can be a source of negative frequency-dependent selection, causing agonistic character displacement and facilitating speciation and coexistence of (sibling) species. In this paper we synthesise the evidence that male-male and female-female competition contributes to cichlid diversification, showing that competition is stronger among same-coloured individuals than those with different colours. We argue that intrasexual selection is more complex because there are several examples where males do not bias aggression towards their own type. In addition, sibling species or colour morphs often show asymmetric dominance relationships. We briefly discuss potential mechanisms that might promote the maintenance of covariance between colour and aggression-related traits even in the face of gene-flow. We close by proposing several avenues for future studies that might shed more light on the role of intrasexual competition in cichlid diversification.

1. Introduction

The cichlid fish in East African lakes are emerging as one of the potentially most powerful model systems in speciation and adaptive radiation research [1–4]. The rock-dwelling communities of these lakes comprise several species complexes or genera that can be strongly differentiated in ecology. By contrast, within genera, sibling species tend to be ecologically more similar, yet strikingly different in male nuptial coloration [5–7]. This interspecific colour variation resembles intraspecific colour variation between hybridising incipient species or colour morphs. The inference is that sexual selection by female mate choice on male colour plays a central role in the evolution and maintenance of haplochromine species richness (e.g., [1, 2, 8, 9]). In haplochromines, male-male competition is likely important since territory ownership is a prerequisite to gain access to spawnings [8, 10]. Moreover, territory quality affects mate choice [11, 12]. Hence, aggressive competition over territory sites is intense and is likely to affect sexual selection. In theory, male-male competition can serve as a source of negative frequency-dependent selection in haplochromine cichlid fish due to stronger competition among same-coloured males than those with different colours [13–15]. The resulting disruptive selection may facilitate several evolutionary processes of diversification, including character displacement [16], reinforcement, speciation [15], and the syntopic coexistence of species [14, 17]. In this paper we discuss the relevance of male-male competition in evolutionary diversification. We summarise the empirical evidence that haplochromine cichlid males bias aggression toward similar-coloured rivals. We then show that negative frequency-dependent selection is often not symmetric and that selection arising from male-male competition is often more complex than previously thought.

2. Relevance of Male-Male Competition in Evolutionary Diversification

If territorial males direct more aggression to rivals that phenotypically resemble themselves than to different phenotypes, rare male varieties would enjoy a frequency-dependent advantage because they receive less aggression [13, 14]. This process is akin to negative frequency-dependent resource competition which provides the disruptive selection that is necessary for many evolutionary models of character displacement and speciation (Figure 1; [18] and references herein).

2.1. Character Displacement. If species are competing for different resources and/or mates, the cost of accidental interspecific aggression may favour the evolution of divergence in competitor recognition traits ("agonistic character displacement", [16]). Before discussing this further, we need to define the different forms of character displacement. "Reproductive character displacement" results from selection against maladaptive interspecific hybridisation resulting in enhanced prezygotic reproductive isolation between sympatric species [19]. If speciation is incomplete, this process is known as reinforcement (see below). It may involve a variety of isolating mechanisms, including enhanced mate recognition by virtue of divergence in male sexual traits. "Ecological character displacement" is defined as shifts in traits relevant to ecological resource use resulting from selection against intermediate "ecotypes" [20].

Agonistic character displacement has received limited attention (e.g., [21, 22]), despite the fact that these ideas date back to the 60s [23, 24]. It can take place in a range of different traits that affect the rate, intensity, and outcome of interference competition [16]. Species that evolved in allopatry and are subsequently brought into sympatry can undergo agonistic character displacement as a result of interspecific aggressive interactions ([13, 25–28], reviewed in [16]).

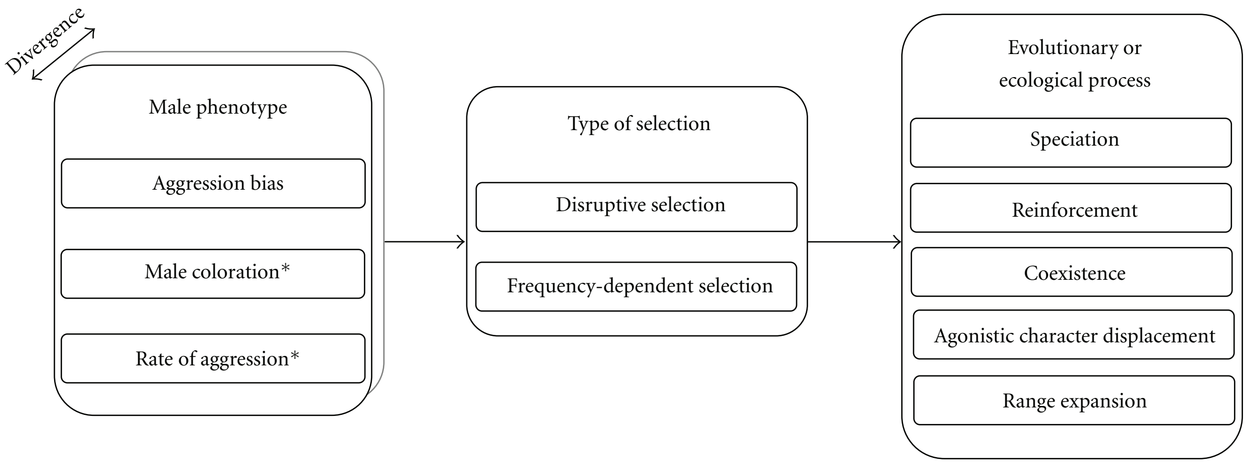
2.2. Speciation. Although male-male competition does not directly contribute to the evolution of assortative mating, it has been implicated as an important factor in the process of speciation by sexual selection since the traits used in mate choice are often also used in intrasexual communication [29]. Male-male competition can contribute to overcoming two important hurdles in models of sympatric speciation by sexual selection (for more details see [15]): (1) considerable genetic variation is required to trigger divergent Fisherian runaway processes [30], but sexual selection may rapidly deplete genetic variability in female mating preferences, hereby constraining sympatric speciation (e.g., [31]); (2) speciation can only occur from a rather narrow range of initial phenotypic distribution of female preference and male display, which must be close to symmetric [15, 32], making the process highly unstable. van Doorn et al. [15] show that speciation can only take place when there is negative frequency-dependent selection on female mating preference assumed possible when males cannot father an unlimited number of offspring, [15, 33, 34] and an additional mechanism that generates negative frequency-dependent selection on either females or males. Since in several taxa male-male competition is a major component of sexual selection [35], aggressive male-male competition for breeding sites could generate this advantage of rarity [15]. Male-male competition may then facilitate the invasion of novel phenotypes, increasing variation in male trait, and stabilising the coexistence of diverging populations and daughter species [15].

2.3. Reinforcement. When species are brought into secondary contact, reproductive isolation can be increased by reinforcement [19]. Speciation by reinforcement is driven directly by selection against maladaptive hybridisation favouring the evolution of nonrandom mating. Hybrids may not be optimally adapted in ecological resource use [19]. Maladaptation can also occur when hybrids incur elevated attacks compared to their nonhybrid counterparts, favouring not only divergence in agonistic signals (agonistic character displacement), but also the evolution of prezygotic reproductive isolation (reinforcement).

2.4. Sympatric Coexistence of Species. Negative frequency-dependent selection arising from male-male competition could also promote species coexistence. Tolerance towards heterospecific neighbours may lead to denser packing of territorial males in mixed species assemblages [14, 36]. In haplochromines, the dense packing of many different species has puzzled many researchers (e.g., [37]), and it has already long been indicated that territoriality can affect the intra- and interspecific interactions that organise spatial patterns of coexistence in communities [36, 38, 39]. In addition, similarity in nuptial dress between resident and immigrant species may determine success or failure of range expansion of the latter in that invading species with dissimilar colour are more likely to be tolerated by the resident species [17, 40, 41].

3. Evidence for Stronger Competition among Same-Coloured Individuals than Those with Different Colours

Several earlier studies indicated that rare male advantages might emerge from intrasexual competition, confirming that same-coloured rival males compete more. Using a large data set of cichlid communities from 47 rocky habitat patches in Lake Victoria, Seehausen and Schluter [13] showed that closely related species with different male nuptial coloration occurred more in sympatry than expected by chance. Also, closely related species of the same colour type were less likely to co-occur than expected by chance. Although disruptive selection on male colour by female mate choice predicts the first pattern [9], it does not predict the negative association between closely related species of the same colour, whereas own-type biases in aggression do. Using abundance data from survey plots of Lake Malawi rock-dwelling cichlids at Thumbi Island, Young et al. [17] found that males of the same body colour had more negative interaction coefficients (derived from a community matrix) than those of different colours. Importantly, they show that male colour influences the abundance and distribution of individuals at the community scale, even among less closelyrelated species. Consistent with this pattern, Pauers et al. [41] provide experimental evidence that males of *Metriaclima mbenjii*, a rock-dwelling species from Lake Malawi, directed more aggressive behaviours towards similarly coloured opponents,



*Affects social dominance

Figure 1: Summary chart of the different components of male-male competition that can generate disruptive and frequency-dependent selection, and the different types of processes it can effect.

regardless of species. This type of selection against coexistence of similar phenotypes regardless of species is also confirmed by aggression experiments in the Lake Malawi cichlid *Metriaclima zebra* (Michael Kidd, personal communication). In field observations Genner et al. [42] showed that territorial male cichlids of the *Pseudotropheus* species complex in Lake Malawi never tolerated males and females of the same species complex in their territories, with a stronger aggression bias for dietary specialists than for dietary generalists, though no colour-based aggression biases were noted. Clearly, aggression can influence the distribution and abundance of haplochromine cichlid species.

There is also evidence for stronger competition among same-coloured rivals at the local (lek) site. First, Lake Victoria and Lake Malawi cichlids have nonoverlapping territories at the intraspecific level, but overlapping territories are more common in males that belong to different species (personal observations, [43]). Second, males of the Lake Victoria cichlid species *Neochromis omnicaeruleus* and *N. rufocaudalis* ("red tail") tend to have territorial neighbours of species that are different in nuptial coloration from themselves [13]. Third, Kohda [36] showed in Lake Tanganyika that the territories of *Petrochromis polyodon* were separated to a greater degree among conspecific males than among heterospecific males. This suggests that territorial males are more tolerant of heterospecific neighbours. In line with this, mesocosm experiments, using the Lake Malawi cichlids *Pseudotropheus emmiltos* and *P. fainzilberi*, showed that the number of territorial males for a given area almost doubled in mixed-species assemblages compared to monospecies assemblages [44]. Not only did heterospecific neighbours receive fewer aggressive interactions than conspecific neighbours, they were also permitted to establish overlapping territories. These findings highlight the potential importance of the increased tolerance for heterospecific males for species coexistence, and it may help explain the syntopic coexistence of different species [42, 45].

Several studies aside from the ones mentioned earlier show that haplochromine males (and females) direct more aggression to conspecifics or same-coloured rivals. Using intruder choice tests, this was demonstrated in several sympatric species pairs from Lake Victoria: *Pundamilia pundamilia* and *P. nyererei* [46, 47], *P. nyererei* and *P. "pink anal"* (for summary see Figure 2), and finally *Mbipia mbipi* and *M. lutea* [48]. The same pattern was demonstrated in two different sympatric species pairs of Lake Malawi cichlids: *Pseudotropheus emmiltos* and *P. fainzilberi*; *P. zebra* and *P. "zebra gold"* [44]. Light manipulation experiments indicate that these aggression biases are largely based on colour differences [44, 46]. Haplochromine females can also behave aggressively [49], and we showed in the Lake Victoria cichlid species with a female colour polymorphism *Neochromis omnicaeruleus* that females bias aggression towards their own morph [50]. This could help stabilise the female colour polymorphism in this species.

If aggression is to facilitate invasion of novel phenotypes (relevant to both speciation and range expansion), it is predicted that males direct less aggression to novel phenotypes. Consistent with this prediction, *Pundamilia* males from a location where most males display blue nuptial coloration directed more aggression towards blue rivals than towards red-coloured *P. nyererei* rivals (Figure 2, [51]). Likewise, in *Pseudotropheus emmiltos* conspecific albino males evoked fewer attacks than "wild type" males [44].

Plenderleith [44] provides evidence for selection against hybrids. In intruder choice tests, hybrid males of *P. emmiltos* and *P. fainzilberi* were treated as a conspecific by males of both parental species as well as any other hybrids. This is consistent with the hypothesis that reinforcement can result from intrasexual selection against hybrids.

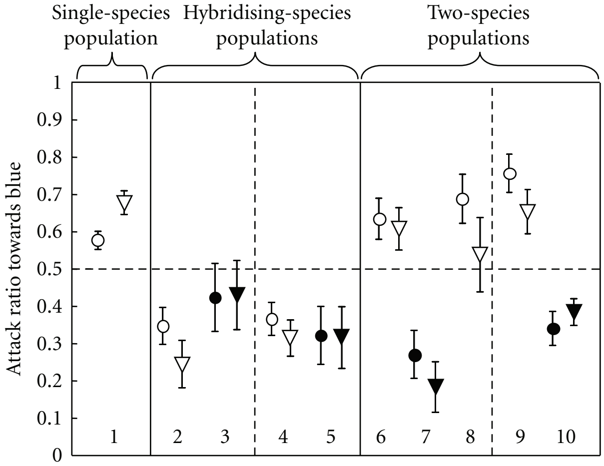


Figure 2: The display and attack ratios of *Pundamilia* males from five separate locations [4]. The different *Pundamilia* phenotypes are indicated below with numbers: the single-species population (1) intermediate between blue and red, mostly bluish, Luanso Island, $N = 28$, the hybridising, incipient species populations (2) blue phenotypes, $N = 7$; (3) red phenotypes, $N = 6$, Kissenda Island Island; (4) blue phenotypes, $N = 22$; (5) red phenotypes, $N = 5$, Python Island Islands and the two-species populations (reproductively isolated sister species, (6) *P. Pundamilia*, $N = 23$; (7) *P. nyererei*, $N = 12$, Makobe Island, (8) P. "pink anal", $N = 10$, Makobe Island; (9) *P. Pundamilia*, $N = 11$; (10) *P. nyererei*, $N = 4$, Senga Point. The response ratio is the response to the blue stimulus divided by the sum of the responses to the red and blue stimuli. A response ratio of 0.5 represents identical responses to the two stimuli (- - -). Symbols indicate mean ± SE values. Black symbols denote red males; white symbols denote blue males. Circles denote display behaviour, triangles denote attack behaviour. Data from [46, 51].

4. Frequency-Dependent Selection Arising from Male-Male Competition Is Not Always Symmetric between Sibling Species

We have summarised the evidence that cichlid males preferentially attack like coloured rival males, likely causing character displacement both within and between species [13, 16] and facilitating speciation and coexistence of different species [14, 15]. However, in the following we argue that selection by male-male competition on colour is more complex than previously appreciated. We first show that males of certain species do not always bias aggression towards their own species, that learning might be involved, and that social dominance effects should also be considered.

4.1. Lack of Own-Type Aggression Bias and Learning. There are several accounts where males do not bias aggression towards their own colour type. First, males of the sympatric species pair *Pundamilia pundamilia* (males referred to as blue) and *P. nyererei* (males referred to as red) from two locations where they hybridise directed more aggression towards red rival males (Figure 2, [46, 51]). The lack of own-type bias in blue males from hybridising species pair in contrast to the behaviour of males where the two species are reproductively isolated can be explained by considering the degree of competition for females (see [46]). Gene-flow between red and blue in the hybridising species pair suggests that males of the red and blue morph compete partly for the same set of females. This may render it less advantageous for blue males to concentrate aggression on males of their own colour than in a situation where red and blue are fully reproductively isolated species. Second, in the Lake Tanganyika cichlid *Astatotilapia burtoni*, males express bright blue or yellow body colours, and males occasionally change colour. Korzan and Fernald [52] found that males preferentially attack rivals with opposite coloration. The final example is from a nonhaplochromine species, the polychromatic, monogamous Central American cichlid *Amphilophus* ("*Cichlasoma*") *citrinellum* (Midas cichlid). In this species individuals start as normally coloured cryptic individuals, but some individuals attain a "gold" coloration. Barlow and Siri [53] show that individuals of both colour morphs behave more aggressively to the "normal" morph. Taken together, these findings suggest that own-type aggression biases can be absent in cases where sibling species hybridise (in the *Pundamilia* system), or where males are conspecific colour morphs (in *A. burtoni* and *C. citrinellum*). This is relevant from an evolutionary perspective, because these findings suggest male-male competition is unlikely to stabilise coexistence of different phenotypes in an incipient stage of speciation, or maintain phenotypic bimodality when closely related species hybridise.

Aggression biases may differ between wild-caught and lab-bred individuals. This is an important consideration since the majority of choice tests are conducted with lab-bred individuals, which may not adequately reflect the situation in the wild. Laboratory-bred blue *Pundamilia* males from a hybridising location biased aggression towards same-coloured rivals [54–56], whereas wild-caught males biased aggression towards red rivals [46, 51]. This may hint at a role of learning in shaping aggression biases in the wild. The occurrence of learning was indeed demonstrated in *Pundamilia* and another cichlid genus, *Mbipia*. Blue *Pundamilia* males raised with only blue males had no preferences for either red or blue rivals, whereas males raised with both blue and red males had a preference for blue rival males, indicating a role of learning [54]. Verzijden et al. [47] found in a crossfostering study that early learning (on the mother's phenotype) does not influence male aggression biases in *Pundamilia* males, in spite of the fact that females can sexually imprint on their mother's phenotype [57]. Aggression biases of males of two other species, *Mbipia mbipi* and *M. lutea*, were affected by the colour of the siblings, but like in *Pundamilia*, not by the colour of the mother suggesting that colour experience may have to be contingent on aggressive interactions [48].

4.2. Dominance Relationship. Asymmetric dominance relationships between sibling species and colour morphs are common in the animal kingdom [58, 59]. This could in theory facilitate the establishment of the more socially dominant phenotype into a given population, which is relevant to both speciation and range expansion. Moreover, asymmetric dominance relationships may also promote agonistic character displacement in the less dominant species or phenotype to avoid costly interspecific interactions (for an example in damselflies, see [24]). Dominance advantages can come about by colour effects on winning dyadic combats and/or intrinsic differences in the rate of aggression.

In the Lake Victoria cichlid fish *Pundamilia* red males were more likely to dominate blue males in dyadic combats, but their advantage was significantly reduced under green light. This suggests the intimidating nature of red coloration [60]. A similar intimidating effect of red coloration has been documented in other animal species [61–64] including cichlid fish (Midas cichlids, *Amphilophus* ("*Cichlasoma*") *citrinellum* [65], firemouth cichlids *Cichlasoma meeki*: [66]). In *Pundamilia* the social dominance advantage of red males is likely not only a colour effect since red males are inherently more aggressive than blue males [46, 55]. The dominance of red is consistent with the geographic distribution pattern of red and blue *Pundamilia* types. Red types always co-occur with blue, whereas purely blue populations are not uncommon [67], indicating repeated invasion of red phenotypes into blue populations by their dominance advantage.

Social dominance is not restricted to the red-blue combination or to males. When *Astatotilapia burtoni* males were allowed to compete physically for the same territory, yellow males became dominant in the majority of trials [52]. In the Convict cichlids *Archocentrus nigrofasciatus* the wild-type (WT) black-barred morph dominates the amelanistic barless morph in dyadic interactions [68]. Finally, in the female colour morphs of *N. omnicaeruleus* white blotched (WB) dominates orange blotched (OB) females, and WB and OB dominate plain colour phenotypes [69].

4.3. Combined Effect of Colour-Related Aggression Bias and Social Dominance. How do colour-related aggression bias and social dominance in dyadic combats jointly influence coexistence between competing species or colour morphs (Figure 1)? We tested this in *Pundamilia* assemblages with different proportions of red and blue males, bred in captivity [56]. We first showed that males of both colour morphs direct more aggression towards rivals of their own type, which is in contrast to the overall aggression bias to red in wild-caught *Pundamilia* males ([46, 51], discussed below). We found that red males were indeed socially dominant over blue ones, but only when rare. However, blue males were not socially dominant when rare. We then tried to disentangle the effects of the own-morph attack bias and social dominance of red using computer simulations. The simulation results suggest that an own-morph attack bias reduces the social dominance of red males when they are more abundant. Although these data suggest that dominance is frequency dependent, we found no evidence of symmetric negative frequency-dependent selection acting on social dominance. These data suggest that male-male competition may contribute to coexistence, but cannot always explain it. Likewise, male-male competition may facilitate speciation, but may also constrain it depending on the shape of frequency-dependent selection.

We have discussed two aggression traits that can exert selection on colour: colour-based aggression biases and social dominance. Little is known about the mechanisms underlying these traits, and it is unclear how the association between colour and aggression traits can be maintained in the face of gene-flow. This will be discussed in the following section.

4.4. Pleiotropy between Colour and Aggression?

It is difficult to understand how own-type biases or differences in rate of aggression can evolve during an initial or incipient stage of speciation. Gene-flow would erode linkage disequilibrium between colour and these aggression traits by recombination, unless, for example, learning or a pleiotropic mechanism links these two traits. This is analogous to a major question in speciation research: does reproductive isolation between populations evolve via one- or a two-allele mechanisms (e.g., [70, 71]). For example, a single allele that increases habitat preference when the diverging species inhabit different environments would make speciation in theory easier because they do not require linkage disequilibria to form as is the case when habitat preferences are described by two different alleles. A type of one-allele mechanism is learning, and as discussed earlier, there is some evidence for learning in *Pundamilia* [54]. Future studies should examine the genetic mechanisms underlying "own-type aggression biases". Here we tested for pleiotropy (or tight linkage) in two polymorphic haplochromine species, *N. omnicaeruleus* and a single-species population of *Pundamilia* (Luanso Island).

We crossed plain with blotched *N. omnicaeruleus*, yielding broods containing both plain and blotched sisters. We found that own-type biases in aggression in these laboratory-bred sisters were broken down, making pleiotropy or tight linkage unlikely explanations for own-type aggression biases in wild-caught females [50]. However, in dyadic encounters, WB female morphs dominated their plain sisters. Given the largely homogenous genetic background of these full sibs, this finding supports the hypothesis that the social dominance of WB females is a pleiotropic effect of colour or that genes coding for colour and those influencing behavioural dominance are genetically linked. This linkage could explain the maintenance of an association between colour and behavioural dominance despite gene-flow. The causal mechanism underlying the link between colour and dominance is an exciting avenue for future research. While we cannot exclude that the more conspicuous colour patterns of WB and OB have an intimidating effect on opponents (*sensu* effect of red), we deem it more likely that an endocrine mechanism link dominance and colour in this species, possibly via the melanocortin system that both modulates skin coloration and a suite of other traits including aggressive behaviour [72].

Also in *Pundamilia* we tested for pleiotropy/tight linkage against the alternative of independently segregating genes. We took advantage of the fact that the *Pundamilia* population at Luanso Island is a single species or hybrid swarm comprised of males showing a continuous distribution of blue to intermediate (between blue and red) phenotypes. We scored the individuals that we tested in Dijkstra et al. [51] using the phenotype scale in Figure 1 [51], and expressed the aggression preference for the blue morph as a function of phenotype score. There was no significant relationship between aggression preference and phenotype score (one-sided Spearman rank correlation: $r = -0.24$, $P > .1$, $N = 28$, making pleiotropy between colour and aggression bias again unlikely. The lack of pleiotropy in polymorphic *Pundamilia* and *N. omnicaeruleus* species suggests that a (strong) buildup of linkage disequilibrium between colour and aggression bias is required for the evolution of own-type biases in aggression. This process is less effective than pleiotropy in causing the expression of own-type biases. Nonetheless, one would predict strong selection favouring individuals that preferentially expel competitors for mates and/or most dangerous usurpers of territorial space, and as suggested earlier, a one-allele mechanism such as learning or other mechanisms during ontogeny might be instrumental in shaping these adaptive aggression biases. Regarding the covariance between colour and intensity of aggression, the possibility that these are pleiotropically linked through a hormonal mechanism deserves more attention, and studies testing the role of the melanocortin system are underway.

4.5. Future Perspectives.

We conclude that the dynamics of frequency-dependent selection arising from male-male competition is probably more complex than previously appreciated (Figure 1). Several components of male-male competition should be considered, such as aggression biases and social dominance asymmetries (Figure 1). Their independent effects can be teased apart by using a combination of community studies and simulations. For example, simulations indicated that the dominance of red *Pundamilia* males over blue males becomes more negative frequency dependent when individuals bias aggression towards their own type [56]. There are several important avenues for future research.

First, to date there is no experimental study in cichlids that actually tested the fitness consequences of frequency-dependent selection arising from male-male competition. Dominant males preferentially occupy high quality territories (size of crevice), and there is some evidence that occupying a high-quality territory translates into reproductive success (e.g., [12]). However, little is known about the long- and short-term fitness consequences of frequency-dependent dominance.

Second, in models of speciation it is usually not taken into account that diverging species may display asymmetries in social dominance. This is an important caveat, since asymmetries in social dominance are common in the animal kingdom, both between conspecific colour morphs and (hybridising) sibling species (e.g., [40, 64, 73]. Differences in success in competition for mates and resources will influence the fitness landscape of diverging phenotypes and will dynamically change the scope and conditions for speciation by disruptive sexual selection. Further, in recent years there is an increase in realisation that natural and sexual selection can jointly drive speciation (e.g., [74, 75], for theoretical paper see [76]) and that in fact sexual traits and preferences are subject to natural selection too (e.g., [75]). Sexual traits may signal local adaptation to females, favouring the evolution of divergent female preferences for these traits. We suggest that frequency-dependent selections arising from male-male competition is also relevant when natural and sexual selection act in concert. Moreover, it is possible that male colour may not signal only local adaptation in ecological traits, but also adaptation in agonistic behaviour tailored to the local "competitive" circumstances.

Third, in competition males do not only pay attention to male body colour, but also other male traits including territory characteristics. Males of rock-dwelling cichlids preferentially occupy larger crevices [12] and more structurally complex territories [77]. In bower-building cichlids bower height may signal dominance over other males, and males placed on shorter bowers were more frequently attacked by neighbouring males [78]. How disruptive selection on colour through male-male competition is influenced by intrasexual selection on other male traits is an exciting topic for future research. For example, rare males may not only be more likely to acquire a high-quality territory, but they may also receive fewer attacks as a result of occupying these high-quality sites, magnifying the rare male effect. By contrast, if high quality territories attract more intense territorial disputes, it would countervail the benefits of occupying a high-quality territory (Machteld Verzijden and Martine Maan pers. comm.).

Finally, both nuptial coloration and aggression have physiological underpinnings. Certain pigments used in nuptial coloration, such as carotenoids, are also required in several health-maintaining functions, posing potential allocation tradeoffs between key life-history traits (e.g., [79]). Agonistic behaviour is regulated by neuroendocrine pathways which impinge on several physiological functions that are important for fitness. A well-known example is the dual effect of androgens in modulating both sexual display and the immune system [80]. This creates a potentially exciting scenario in which colour morphs differ not only in androgen levels, but also in life-history tradeoffs. For example, in *Pundamilia* red and blue males differed in immunity and oxidative stress (Dijkstra, unpublished). In *Astatotilapia burtoni* yellow males had significantly higher levels of 11-ketotosterone than blue males [73]. Indirect disruptive selection on hormones (via for example selection on territoriality) may thus facilitate or constrain adaptive evolution in correlated traits (see e.g., [81]). Integrating physiology with evolutionary biology might therefore yield more insight into the mechanisms of phenotypic diversification in haplochromines.

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Research Article

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

Ola Svensson,[1,2] Bernd Egger,[3] Boye Gricar,[1] Katie Woodhouse,[1,4] Cock van Oosterhout,[1,5] Walter Salzburger,[3] Ole Seehausen,[6,7] and George F. Turner[1,8]

[1] *Evolutionary Biology Group, Biological Sciences, University of Hull, Hull HU6 7RX, UK*
[2] *Department of Zoology, University of Gothenburg, P.O. Box 463, 405 30 Gothenburg, Sweden*
[3] *University of Basel, Zoological Institute, Vesalgasse 1, 4051 Basel, Switzerland*
[4] *Easton College, Easton, Norwich, Norfolk NR9 5DX, UK*
[5] *School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, UK*
[6] *Swiss Federal Institute of Aquatic Science and Technology (Eawag), Centre for Ecology, Evolution & Biogeochemistry, Seestrasse 79, 6047 Kastanienbaum, Switzerland*
[7] *Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, 3012 Bern, Switzerland*
[8] *School of Biological Sciences, Bangor University, Gwynedd LL57 2UW, Wales, UK*

Correspondence should be addressed to Ola Svensson, ola.svensson@zool.gu.se

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

1. Introduction

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

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

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

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

2. Methods

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

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

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

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

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

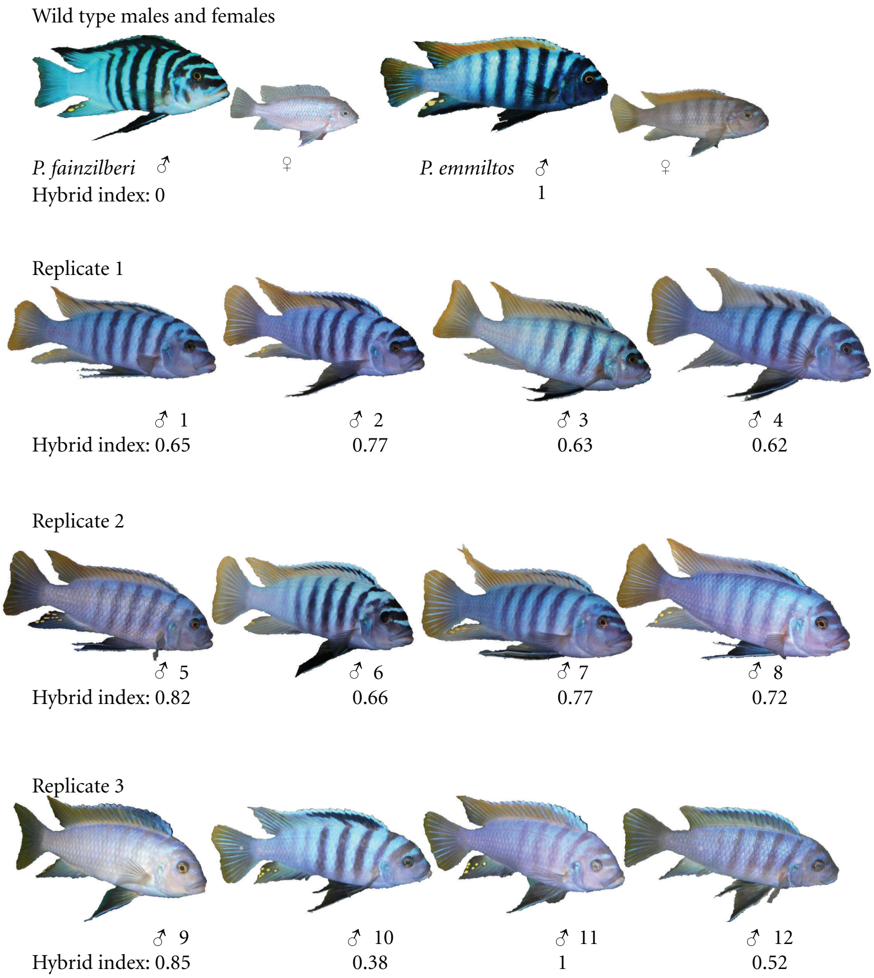


Figure 1: Wild-type males and females and second-generation hybrid males. Wild-type male and female *Pseudotropheus fainzilberi* and *P. emmiltos* from Mphanga Rocks, Lake Malawi, as well as the twelve second-generation (F_2) male hybrids used in replicates 1–3. The hybrid index was based on variation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

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

2.4. Testing Spawning Decisions. A randomisation procedure was used to test the null hypothesis that males were equally attractive to *P. fainzilberi* and *P. emmiltos* females. We used a Monte Carlo method with 1000 simulations per replicate to simulate the spawning decisions of a total of 44 females (i.e., 15 *P. emmiltos* and 29 *P. fainzilberi* females) with 12 F_2 hybrid males (in three replicates). We calculated the probability of finding a combination of spawning decisions that is more uneven than the one observed, assuming that all males were equally attractive. The expected number of spawnings with male Mi by *P. fainzilberi* (Pf) females was therefore, calculated as $N(\mathrm{Mi} \times \mathrm{Pf}) = N_{\mathrm{Mi}} N_{\mathrm{Pf}} / (N_{\mathrm{Pf}} + N_{\mathrm{Pe}})$, where N_{Mi} is the total number of spawnings received by male (Mi), N_{Pf} is the total number of spawnings summed across all *P. fainzilberi* (Pf) females in the replicate, and N_{Pe} is the total number of spawnings by all *P. emmiltos* females in the replicate. The expected number of spawnings of *P. emmiltos* (Pe) females with male Mi was calculated in the same manner. We randomised the females, allocating the observed total number of spawnings to each male, and then calculated the absolute deviation between the mean expected values and each of the simulations (Δsim). This simulated distribution of deviations was then used to statistically test the deviation between the observed and the expected values of the numbers of spawnings for each male (Δobs). This procedure tests whether the relative preference for a particular male differs significantly between the two species of females. The macro for this procedure was written in Minitab 12.1 and is available from the authors upon request. In addition, we tested the spawning decisions for each male separately using a binomial equation [22]. The probability of spawning of a Pe and Pf female was equal to their relative proportions in the replicate.

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

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

3. Results

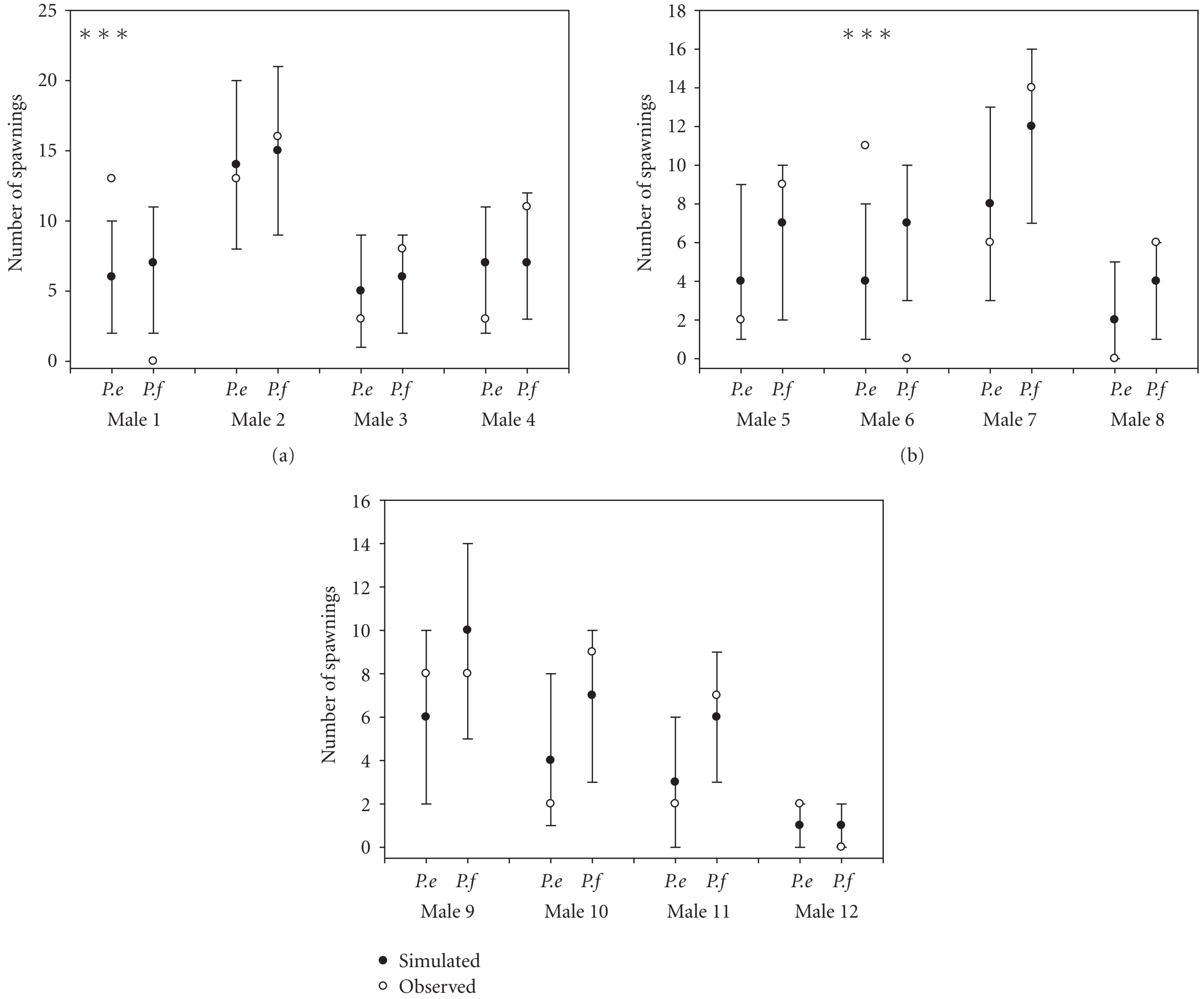
We successfully determined paternity for 395 out of 398 embryos. By comparing the simulated with the observed data we rejected the hypothesis that all males are equally attractive to females of the two species in replicate 1 ($P = .011$) and replicate 2 ($P = .001$), where males were allocated randomly,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

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

4. Discussion

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

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



(a)

(b)

(c)

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

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

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

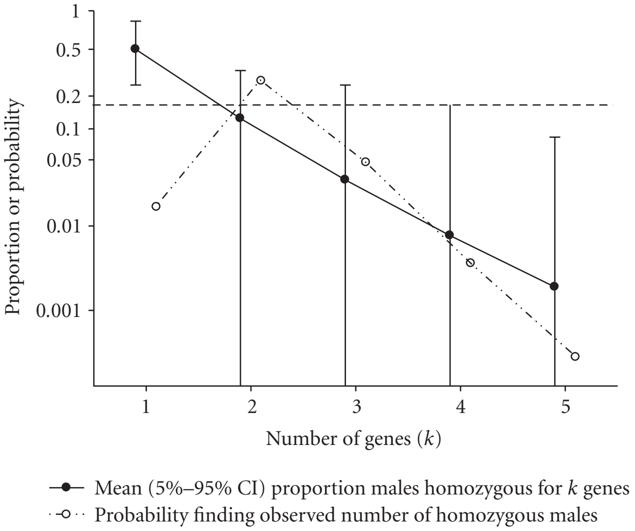


Figure 3: Result from the quantitative genetic model. Results from the quantitative genetic model and observations. Mean (±5–95% CI) expected proportion of F_2 males that are completely homozygous for the reproductive isolation genes (k) of one of the species (solid symbols and line). The open symbols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

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

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

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Research Article

One Fish, Two Fish, Red Fish, Blue Fish: Geography, Ecology, Sympatry, and Male Coloration in the Lake Malawi Cichlid Genus *Labeotropheus* (Perciformes: Cichlidae)

Michael J. Pauers

Section of Vertebrate Zoology, Milwaukee Public Museum, 800 West Wells Street, Milwaukee, WI 53233, USA

Correspondence should be addressed to Michael J. Pauers, mjpauers@gmail.com

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While sexual selection on male coloration has been important in haplochromine cichlid speciation, few studies to date have examined potential environmental influences on color pattern evolution. Data from multiple sources on male nuptial coloration of the Lake Malawi endemic genus *Labeotropheus* were used to examine the relationship between color patterns and the environments in which these patterns were found. Red- or carotenoid-pigmented males were concentrated in the northwestern portion of Lake Malawi and were also associated with increasing depth. Further, the presence or absence of *L. fuelleborni* influenced the coloration of *L. trewavasae* populations; when *L. fuelleborni* was present, *L. trewavasae* males were more likely to exhibit some degree of red coloration. While these results support the idea that sexual selection on male coloration is an important factor in the haplochromine speciation, they also underscore the importance of environmental influences on the evolution of color patterns.

1. Introduction

An organism's color can serve an adaptive function in numerous ecological contexts, including crypsis, communication, and thermoregulation [1, 2]. As such, it is likely that organismal color reflects a balance among numerous and perhaps competing demands; a color best suited for the performance of one function (e.g., cryptic avoidance of predators) may reflect a trade-off with that suited for another (e.g., attractiveness to potential mates). This trade-off is further shaped by the wavelengths of light available in the organism's natural environment; a color pattern that is cryptic in one environment may be conspicuous in another [1, 3–5]. In other words, the relative strength of the color signal depends on not only the visual system of the receiver but also the medium and surrounding environment in which it is transmitted [3, 6, 7].

This trade-off is especially important in aquatic environments, where the available wavelengths of visible (and near-visible) light are highly habitat dependent, thus potentially placing a limit on the palette of colors available to the organisms that live there [7–10]. The background against which the color patterns of aquatic organisms are typically viewed will also further constrain the efficiency of certain visual signals [2, 4, 7, 8, 11]. Because of these limitations on the spectral environment of aquatic systems and their potential effects on organismal color, aquatic organisms are excellent choices for studying the interactions of environment or ecology and the evolution of color patterns.

The bright colors of male haplochromine cichlids have received much attention from evolutionary biologists. These colors caused early researchers to speculate that sexual selection may have played an important role in African cichlid speciation [12, 13], an idea that was later confirmed in several lab and field studies [14–17]. Indeed, it has even been proposed that sexual selection via female choice is solely responsible for the evolution of these color patterns, with little or no role allowed for the effects of ecological and environmental context [18, 19]. And, despite the early attention paid to female choice, several researchers found evidence that male intrasexual interactions may play a role in the diversification of color patterns among haplochromine species. Pauers et al. [17], working with several Lake Malawi species, performed a laboratory study in which they found that males were more aggressive towards similarly colored opponents, whether or not they were of the same genus. Further, Seehausen and Schluter [20] and Young et al. [21] found that the membership of communities of Lake Victoria and Lake Malawi species was dictated by male coloration; in both lakes, communities of cichlids were more likely to be composed of species in which the males were differently colored from one another.

Despite this, the evidence regarding the relationship between color (or color pattern) and environment has been equivocal. For example, Goldschmidt [22] demonstrated that the size of anal fin egg spots and, to a lesser extent, egg spot number, increase with decreasing light intensity in Lake Victorian haplochromines. McElroy et al. [23] and Deutsch [18] took spectral measurements from published pictures of Lake Malawi haplochromines and looked for patterns among coloration and environment. While Mc Elroy et al. [23] found some statistically significant correlations, mostly in terms of substrate preference and diet, Deutsch [18] found no significant relationships between male color and ecology, attributing color diversity instead to sexual selection via female choice. Seehausen et al. [19] examined gross color pattern type among all lineages of African cichlids and mapped these color characteristics on a phylogenetic tree. The resulting phylogenetic associations between color pattern elements and ecology indicated that while various stripe patterns had strong ecological associations, male nuptial coloration concomitantly originated with the evolution of promiscuous mating systems in East African cichlids; further, male nuptial coloration may have evolved in a direct response to female mate choice.

Additionally, two more recent, largely field-based studies also drew contrasting conclusions regarding the relationships among male nuptial coloration and the spectral environment. Seehausen et al. [24] found that at populations of a polychromatic Lake Victorian haplochromine in which there was a moderate spectral gradient between shallow and deep habitats, there was a distinct gradient of male coloration, with blue males inhabiting the shallows and red males living at deeper depths. On the other hand, at populations in which there was a steep spectral gradient, the variation among nuptial color types was muted and no clear pattern was found. Interestingly, patterns of female mate choice closely followed these patterns of male nuptial color. Dalton et al. [25] found that among cichlid species within a single Lake Malawi location, there was no relationship between any aspect of male coloration and any feature of the environment, including depth.

Further complicating our understanding of the relationship between color and environment is the relationship between cichlid visual sensitivity and the environment. In an important early study, Muntz [26] found that Lake Malawi cichlids had different complements of visual pigments depending upon the depths at which they live. This was confirmed for African cichlids in general by Spady et al. [27], who found that cichlids from clear habitats expressed different photopigments or differently tuned versions of the same photopigments; this was also found in Lake Victorian cichlids by Terai et al. [28] and Carleton et al. [29]. Furthermore, there is evidence that turbidity constricts male cichlid coloration to those colors that best match or contrast with the surroundings. Lake Malawi is a clear, bright lake and tends to have fish with predominantly blue and yellow coloration. Lake Victoria, being more turbid, tends to have more fish with red and green pigments [18, 23–25]. Given this, it is reasonable to expect that changes in light at various depths could constrain male nuptial coloration (but see Dalton et al. [25]).

The African cichlid genus *Labeotropheus* (Ahl 1927; [30]) is one of the rock-dwelling haplochromine "mbuna" endemic to Lake Malawi. It contains two recognized species, *L. fuelleborni* and *L. trewavasae*. *L. fuelleborni* is a deep-bodied, shallow-dwelling algae scraper often found in association with large rocks, while *L. trewavasae* is a slender-bodied, deeper-dwelling algae scraper that prefers the bottoms and sides of smaller rocks [31, 32]. Despite these differences in ecology, the two species share a cosmopolitan distribution throughout the lake, as well as differing male nuptial colors among populations [31–34]. Further, while *L. fuelleborni* and *L. trewavasae* may be found at the same location, the males of these species very rarely have similar nuptial colorations when found in sympatry [33, 34]. As with many other mbuna populations, *Labeotropheus* populations are isolated from each other by a combination of habitat discontinuities and behavioral mechanisms, so it is likely that there is very little, if any, gene flow among them [31, 32, 35].

Lake Malawi is a large and heterogeneous lake, which suggests that isolated populations of *Labeotropheus* may experience unique visual environments that could influence the evolution or development of their visual systems [24, 31, 36]. Since both intra- and intersexual selection have been demonstrated to play important roles in the evolution of male nuptial coloration in the mbuna in general and in the *Labeotropheus* in particular [15, 16, 37], differing spectral environments among populations could, in turn, have an effect on male nuptial coloration. Thus, the analysis described below is an attempt to explore possible relationships among environmental features of *Labeotropheus* populations and male nuptial coloration and to explore the relative contributions of natural and sexual selection to the evolution of male color patterns in the *Labeotropheus*.

2. Methods

2.1. Data Collection. Pictures or descriptions of *Labeotropheus* color patterns were obtained from six sources [31–33, 38–40]. The information from all five sources was sufficient to classify each *Labeotropheus* populations as one of Seehausen et al.'s [41] three general haplochromine nuptial color pattern types: Blue, Red Dorsal, or Red Ventral (Table 1). In applying these color categories to Lake Malawi cichlids, it should be noted that the adjective "red" refers to carotenoid pigmentation in general, including hues of orange, yellow, and ochre, as there are very few examples of pure red coloration in these fishes [23, 41]. In addition, I further classified fish based on the pigment ("Red" or Blue) that colored the majority (i.e., greater than 50%) of the body of the fish, excluding the fins. This was done for two reasons. First, I wanted to separate coloration that might be more influenced by environmental factors (i.e., the trunk of the fish) from that which might be more influenced by female mate choice (i.e., the fins). For example, a common color pattern in Lake Malawi cichlid fishes is a version of Red Dorsal in which the entire body of the fish is blue and only the dorsal fin is red (Table 1). While swimming, with the median fins clamped closely to the body, this fish may appear to other organisms to be entirely blue; however, when engaged in display activity, whether warding off a competitor or attempting to attract potential mates, its full color pattern is visible. Secondly, I wanted to control for the degree to which a particular population expresses one of these nuptial color types (e.g., both a blue-bodied fish with a red dorsal fin and a red-bodied fish with a white belly are considered Red Dorsal under this classification; Table 1).

Two sources [31, 32] provided information on the minimum, modal, and maximum depths observed, gross habitat type (mainland, island, or rock/reef) and latitude/longitude for some populations of *Labeotropheus* ($n = 35$). Konings [39] was useful in determining some habitat types and latitude/longitude for some additional populations ($n = 36$). For populations where direct information was not available, depths were estimated based on species and habitat type. These depth estimations are reasonable because Ribbink et al. [32] demonstrate that *Labeotropheus* populations tend to have characteristic depth distributions based on both species and gross habitat type. Mainland populations of either species tend to be shallower than island populations, which are shallower than rock/reef populations (see [32, Figure 3, Page 228]). Therefore, those populations for which depth information was unavailable ($n = 43$) were assigned the mean values of minimum, modal, and maximum depths for populations of the same species occurring at the same type of habitat, making habitat type a satisfactory surrogate for depth.

It should be further noted that, in the descriptions and discussions that follow, when I refer to any category (minimum, maximum, or modal) of depth, what I am actually referring to is the depth at which *Labeotropheus* are found.

Data were compiled with separate entries for each species at each location. The data included information on male nuptial, body color, and environmental information (depth, latitude, longitude, habitat type, and whether one or both species of *Labeotropheus* were present at a given location).

2.2. Statistical Analyses. The data were analyzed using a multivariate general linear model in the Set Correlation module in Systat (v. 10.0) [42, 43]. The data were subdivided into two distinct sets; data regarding the fish themselves (species, nuptial color, and body color) were considered to be one set, while the environmental parameters (depth, latitude, longitude, habitat type, and presence of one or both *Labeotropheus* species) were considered to be another. Using the fish data as a set of dependent variables and the environmental data as a set of independent variables, I looked for patterns of correlation and predictability both within and between these sets with Set Correlation. The Set Correlation module provides two types of output: (1) basic correlations among variables both within and between sets and (2) a regression of the independent set against the dependent set, in order to find possible predictive relationships among the variables. Further, a categorical variable with n possible states was collapsed into n-1 factors using a Canonical Correlation Analysis [43]; thus, nuptial color, with three possible states (Blue, Red Dorsal, and Red Ventral), was collapsed into two factors, identified as Nuptial Color (1) and Nuptial Color (2), respectively.

Within the independent set, habitat type was collapsed into two distinct habitat factors based on differences in *Labeotropheus* depth and geographical distribution. While Systat does not identify the particular habitat most strongly associated with these factors, examining the depth and geographical factors that have the highest correlations with each habitat can identify the habitats designated as Habitat (1) and Habitat (2), respectively; this same approach can identify the dependent factors as well. In the dependent set, nuptial color yielded two separate nuptial color factors and body color was reduced into a single factor. Further, the two species of *Labeotropheus* were similarly reduced to a single species factor.

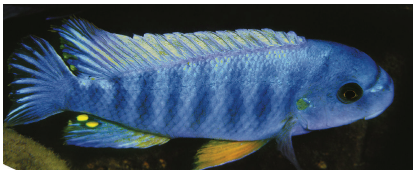
Correlations between the dependent and independent sets were used to determine which color patterns were associated with which environmental variables. Identification of predictive relationships between sets was accomplished by evaluating the standardized regression coefficients and their respective P values for relationships among variables between the dependent and independent sets. Canonical correlations between sets were generated from the rotated canonical factors for both the independent and dependent sets.

Because of the estimation process used to generate some of the depth data, I used the semipartial correlation of known versus estimated depth data type with the independent set of variables, yielding an X semipartial correlation [43]. Partialling, which is a generalization of residualization procedures common to multiple regression and correlation, allows one or more variables to be "partialled out" of either (or both) the dependent or independent sets, thus creating a new set (or sets) in which the variables have zero correlations with the original set (or sets) [42]. This procedure helps to minimize any possible spurious effects of the estimation of depth data and has the further effect of reducing error variance, thus increasing statistical power [42, 43]. In this case, the Systat created a dummy variable for depth data type, giving a value of 1 or 0 for known or estimated data, respectively, and then performed the partialization on the independent set [43]. Thus, partialling the independent set by the type of depth data removed any effects of estimating the depths of the various *Labeotropheus* populations.

3. Results

The overall analysis is significant and robust (Rao's $F_{32,\,219.2} = 5.459$; $P \leq .001$) and only slightly affected by shrinkage ($R^2 = 0.881$; shrunk $R^2 = 0.803$).

Table 1: Possible nuptial color and body color combinations in Labeotropheus. Photos generously provided by Ad Konings/Cichlid Press.

Nuptial color pattern	Possible body color	Example	Number of populations	
			L. fuelleborni	*L. trewavasae*
Blue	Blue		25	14
Red Dorsal	Blue		4	8
	Red		1	10
Red Ventral	Blue		1	0
	Red		5	4

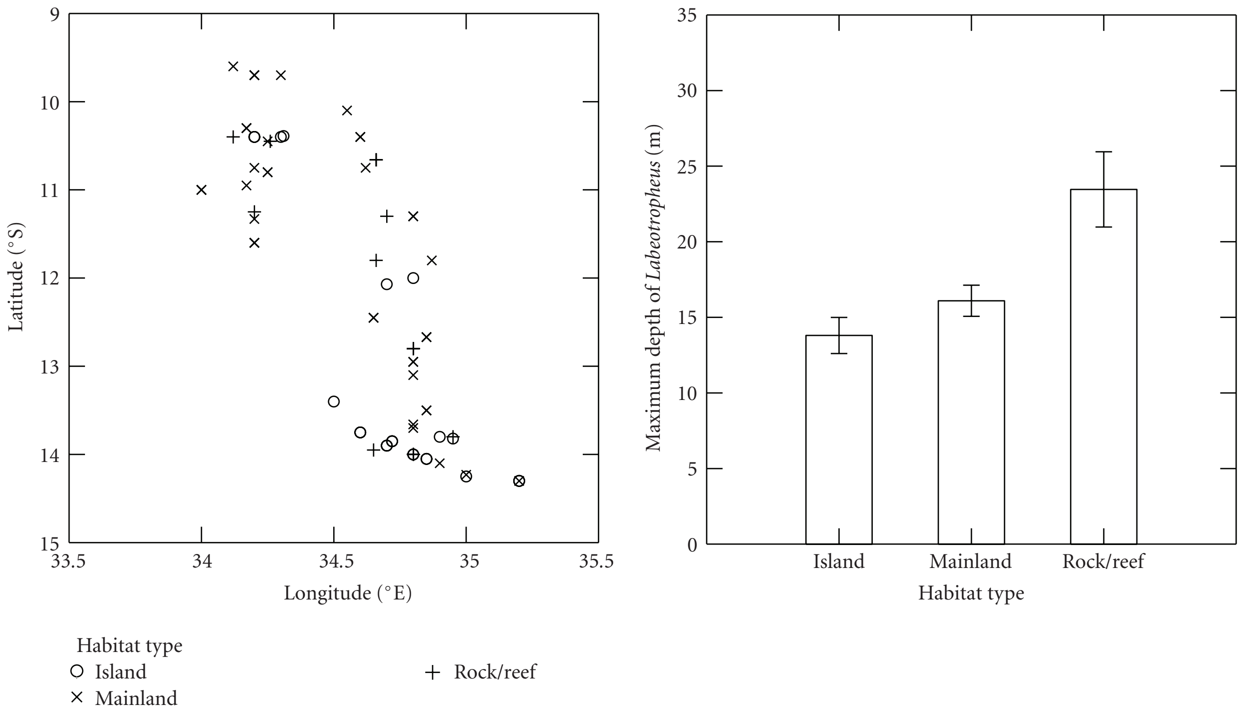
3.1. Within-Independent Set Correlations: Habitat Type Discrimination. The within-independent set correlations are presented in Table 2. Habitat (1) is moderately and negatively correlated with latitude and longitude and strongly and negatively correlated with minimum depth, suggesting a habitat type with a northwestern bias in its distribution at which *Labeotropheus* are found at relatively shallow depths. Figures 1(a) and 1(b) demonstrate that mainland populations of *Labeotropheus* are more prevalent in the northern and northwestern portion of the lake than island or rock/reef habitats and that, as found by Ribbink et al. [32], these mainland populations have the shallowest distributions of *Labeotropheus*; thus, Habitat (1) is indicative of mainland habitats. Habitat (2) has strong, positive correlations with all three measures of *Labeotropheus* population depth. This association with depth suggests that the habitat with the deepest distribution of *Labeotropheus* is identified by Habitat (2); therefore, this factor identifies rock/reef habitats (Figure 1(b)).

Table 2: Identification of habitat type (Mainland, Island, or Rock/Reef) from within-independent set correlations; informative values bolded.

	Habitat (1)	Habitat (2)
Latitude	**−0.279**	0.050
Longitude	**−0.226**	0.050
Minimum depth	**−0.471**	**0.549**
Maximum depth	−0.127	**0.435**
Modal depth	−0.237	**0.550**
Number of *Labeotropheus* species	0.008	−0.06

3.2. Within-Dependent and Between-Set Correlations: Identification of Species and Color Pattern. The identification of species, nuptial color patterns, and body color identified by the Set Correlation procedure requires both the within-dependent and between-set correlations, as some of the correlations for a given factor are stronger in one type of correlation versus the other; this is especially the case with the nuptial color factors.

Table 3 contains the between-set correlations, which are helpful in identifying both the species and body color factors. The species factor, Species (1), shows a strong, positive association with depth; thus, *L. trewavasae* is the *Labeotropheus* identified by this factor. Body Color (1) is



(a)

(b)

Figure 1: Within-independent set correlations and habitat type identification. (a) Geographic distribution of habitats at which *Labeotropheus* populations are found in Lake Malawi. (b) Maximum depths at which *Labeotropheus* populations are found at each habitat type.

strongly and negatively correlated with both longitude and latitude, suggesting a northwestern bias in the distribution of this coloration. Red body color displays this kind of geographic distribution (Figure 2(a)); thus, Body Color (1) represents Red body coloration.

Both nuptial color factors have slight to moderately negative correlations with longitude and latitude, suggesting a northwestern bias in their distributions; this is especially the case for Nuptial Color (2) (Table 3). Also, as shown in the within-dependent set correlations (Table 4), both have a strong correlation with Body Color (1), suggesting that they are comprised of mostly carotenoid pigmentation. Further, the strong correlation between Nuptial Color (1) and Species (1) indicates that it is most common in *L. trewavasae*. As shown in Figure 2(b), the most common male color pattern in *L. trewavasae* is Red Dorsal; thus, Red Dorsal is the color pattern identified by Nuptial Color (1). Figure 2(c) displays the geographic distributions of *Labeotropheus* color patterns, and the pattern with the most distinct northwestern bias in its distribution is Red Ventral; Nuptial Color (2) identifies the Red Ventral pattern.

3.3. Regression Analysis: Predictive Relationships. The results of the regression analyses show that Species (1), Nuptial Color (1), and Body Color (1) have significant relationships predicted by at least one of the independent variables; none of the relationships involving Nuptial Color (2) and any of the independent variables are significant (Table 5). Species (1), which represents *L. trewavasae*, has significant relationships with all measures of depth and Habitat (2). The signs of the coefficients for minimum depth and Habitat (2) are negative, while those for maximum depth and modal depth are positive, suggesting that *L. trewavasae* is found most often at deeper, mainland habitats (Figure 3(a)).

Body Color (1), representing Red body coloration, also has a significant and positive relationship with depth (Figure 3(b)). Further, Body Color (1), as the between-set correlations above demonstrate, has a significant, negative relationship with latitude, suggesting a northern bias in its distribution (see Figure 2(a)). Nuptial Color (1), representing the Red Dorsal nuptial color pattern, has significant, positive relationships with maximum depth (Figure 3(c)) and with the number of *Labeotropheus* species present at a given location (Figure 4).

4. Discussion

The set correlation analysis performed on the *Labeotropheus* biogeographical dataset recovered several correlations between coloration and environment, as well as some predictive relationships among the color and environmental variables. The correlations found included correlations between *L. trewavasae* and depth, Red Dorsal nuptial coloration and depth, and a correlation between a northwestern geographical distribution and both Red Ventral nuptial coloration and Red body coloration. The regression analysis

Table 3: Between-set correlations; informative values bolded.

	Species (1)	Nuptial Color (1)	Nuptial Color (2)	Body Color (1)
Latitude	−0.247	−0.039	**−0.340**	**−0.466**
Longitude	−0.200	−0.060	**−0.306**	**−0.383**
Minimum depth	0.308	−0.074	−0.056	−0.082
Maximum depth	**0.768**	**0.267**	**−0.161**	0.135
Modal depth	**0.737**	0.155	−0.095	0.029
Habitat (1)	0.126	0.089	0.049	**0.249**
Habitat (2)	0.097	−0.003	−0.101	−0.171
Number of *Labeotropheus* species	0.007	0.190	−0.118	−0.049

Table 4: Within-independent set correlations; informative values bolded.

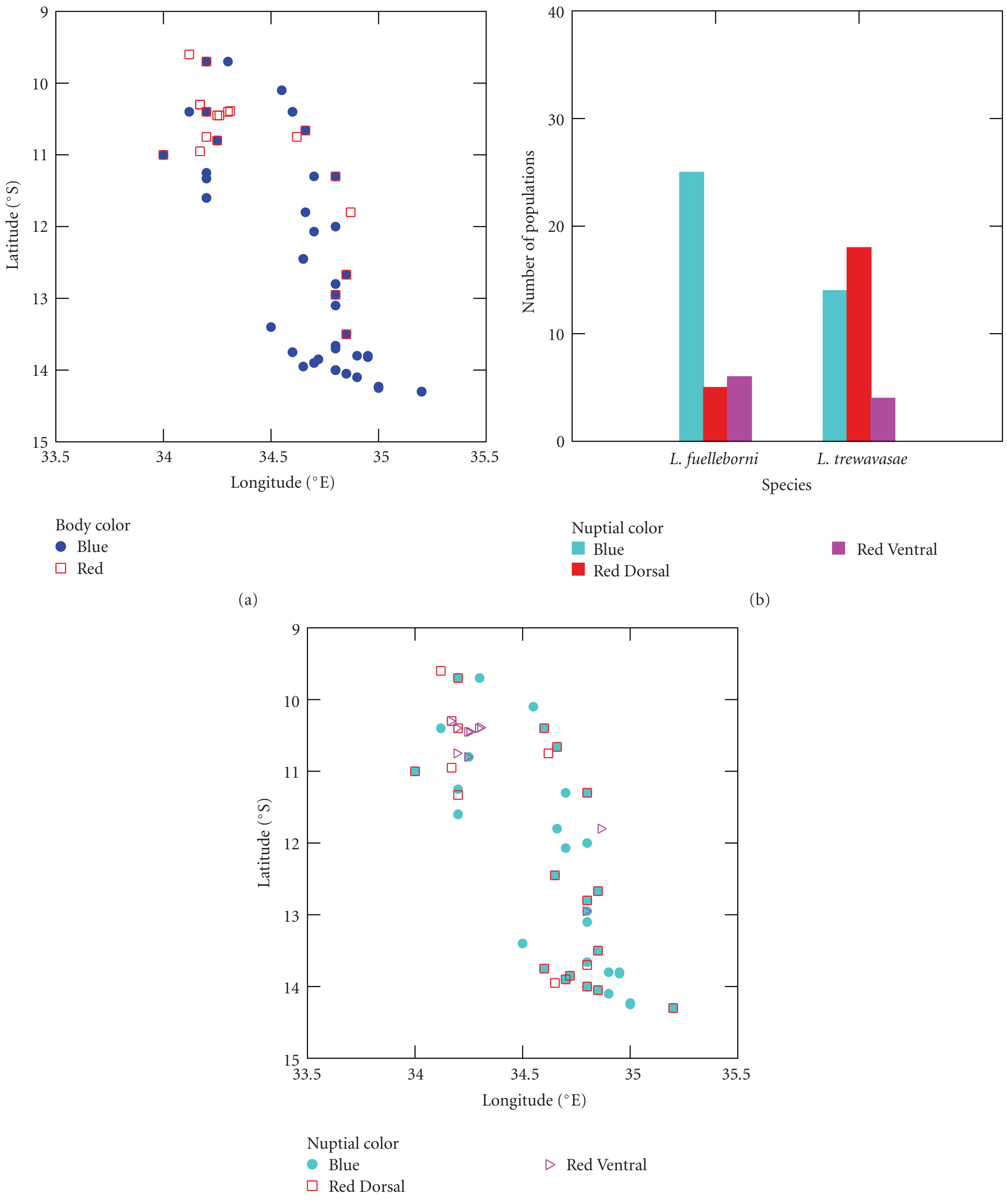
	Species (1)	Nuptial Color (1)	Nuptial Color (2)	Body Color (1)
Species (1)	1.000			
Nuptial Color (1)	**0.387**	1.000		
Nuptial Color (2)	−0.080	−0.275	1.000	
Body Color (1)	**0.248**	**0.307**	**0.558**	1.000

also recovered the relationships between *L. trewavasae* and depth, as well as those between both geography and depth and Red body coloration. The most interesting result of the regression analysis, however, is the predictive relationship between Red Dorsal nuptial coloration and sympatry of the *Labeotropheus* species; when found at the same location, the males of the deeper-dwelling *L. trewavasae* adopt a Red Dorsal nuptial color pattern.

It is not surprising that this analysis recovered the well-documented relationship between *L. trewavasae* and depth; *L. trewavasae* is found most frequently at and prefers water depths of typically more than 15 meters [32]. On the other hand, the disjoint geographical distribution of color patterns in *Labeotropheus* is interesting, with red-pigmented populations concentrated in the northwestern portion of Lake Malawi; a similar pattern of geographic bias in male nuptial color types has been found in the *Metriaclima* [44]. This suggests two possible explanations. First, it may be that natural or sexual selection favors red-/carotenoid-pigmented individuals in the northwest part of the lake; the spectral properties of the water and backgrounds in this area of Lake Malawi could possibly favor reddish coloration for crypsis or communication. There is some corroborating evidence for this possibility, as the northern portion of Lake Malawi experiences a significant amount of rainfall, so the nearshore waters here could be more turbid than in other parts of the lake (H. Bootsma, pers. comm.). Thus, the abundance of reddish *Labeotropheus* in this region would be expected, as African cichlids in more turbid environments display more red coloration in their nuptial coloration [18, 23]. A second possibility for the disjoint geographical distribution of color patterns in *Labeotropheus* is that the populations clustered within either the northwestern or more southern regions of the lake could be more closely related to each other than to populations from the other region. Recent investigations of genetic relatedness among populations suggest that this may be the case [35, 44], though there is some evidence to the contrary [44, 45]. In order to address these two hypotheses regarding the distribution of male coloration, investigations of habitat light and background color (e.g., [8, 25]), and geographic patterns of genetic relatedness (sensu [35]), should be carried out on the *Labeotropheus* populations in these portions of Lake Malawi.

Further, the regression analysis revealed that Red Dorsal nuptial coloration is also significantly and positively related with increasing maximum depth, as was Red body coloration. Red body coloration was also found to have a significant, negative relationship with latitude, suggesting that it has a northern bias in its distribution in Lake Malawi. While the implications of a geographical bias in the distribution of male nuptial coloration in the *Labeotropheus* were discussed in the preceding paragraph, the association between color and depth merits further consideration. Lake Malawi is a very clear lake; even so, long wavelength light acutely attenuates between 0 and 10 meters of depth, which is much shallower than the maximum depths recorded for populations of *L. fuelleborni*, as well as the maximum or modal depths of many populations of *L. trewavasae* [31, 32]. Thus, it seems paradoxical that fish with red or orange coloration should be found where the strength of this signal would be constrained. Recent research by Dalton et al. [25] suggests that, despite the limited amount of long-wavelength light at these depths, the mbuna visual system allows for a surprising degree of color constancy, thus seeming to permit red- or carotenoid-pigmented fish to live in areas where their coloration would otherwise be obscured. Further, Dalton et al. [25] found that in one population of *L. trewavasae*, the coloration of both male and female fish is conspicuous against all possible backgrounds in their environment, suggesting that the saturation of colors on the fish may also aid in their visibility.

Perhaps the key finding of the regression analysis is the significant, positive relationship between the number of species of *Labeotropheus* present at a given location and



(a)

(b)

(c)

Figure 2: Between-set correlations and identification of nuptial and body color factors. (a) Geographic distribution of *Labeotropheus* body color types in Lake Malawi. (b) Frequency of nuptial color types in *L. fuelleborni* and *L. trewavasae*. (c) Geographic distribution of *Labeotropheus* nuptial color types in Lake Malawi.

Red Dorsal male nuptial coloration. When both species of *Labeotropheus* are present, one of them adopts a Red Dorsal color pattern. Interestingly, as shown in Figure 4(b), it is the deeper-dwelling *L. trewavasae* that adopts this pattern when found in sympatry with *L. fuelleborni*. While it has been previously noted that, when sympatric, the *Labeotropheus* tends to adopt different nuptial coloration [33]; this is the first time this relationship has been confirmed quantitatively.

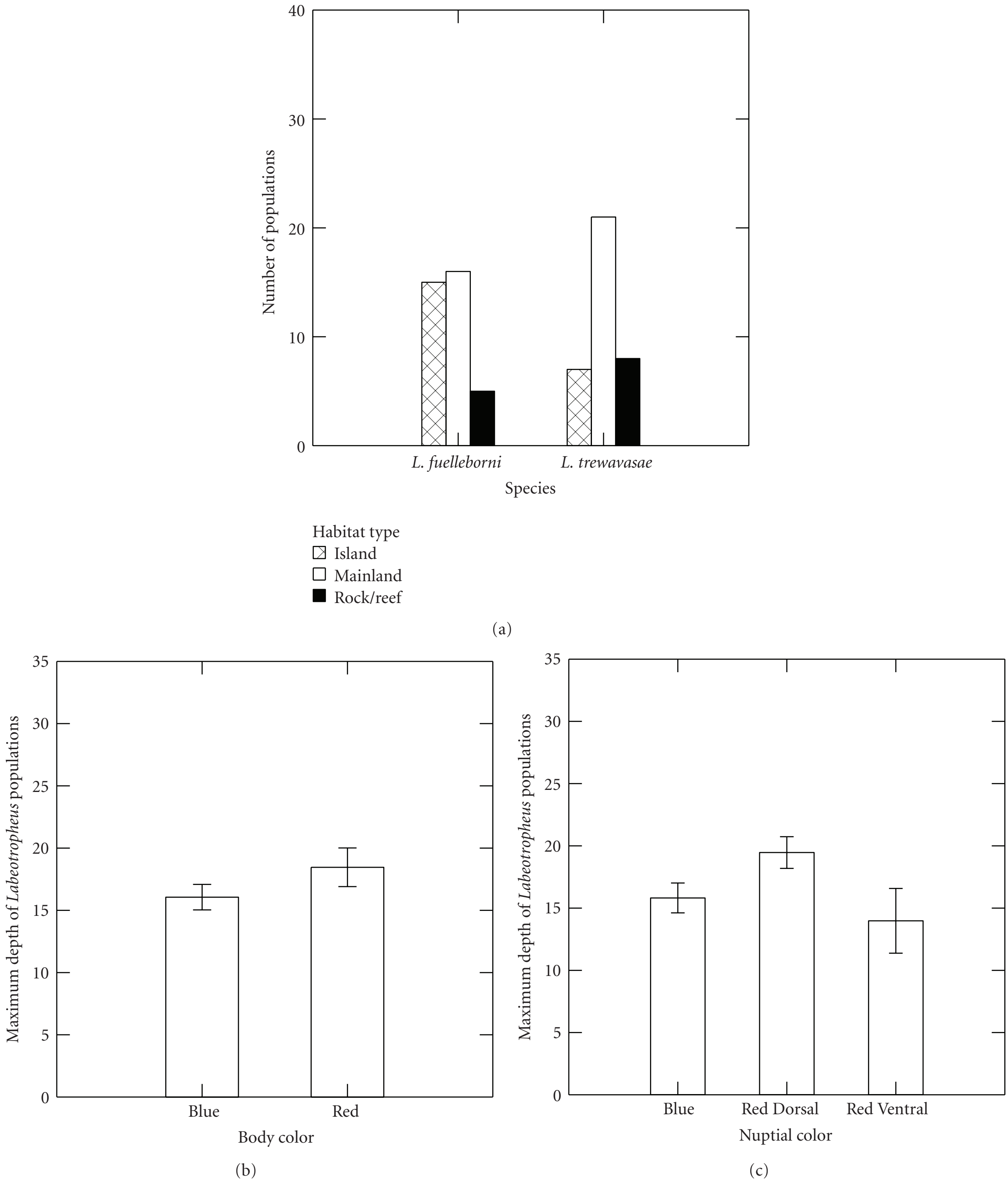
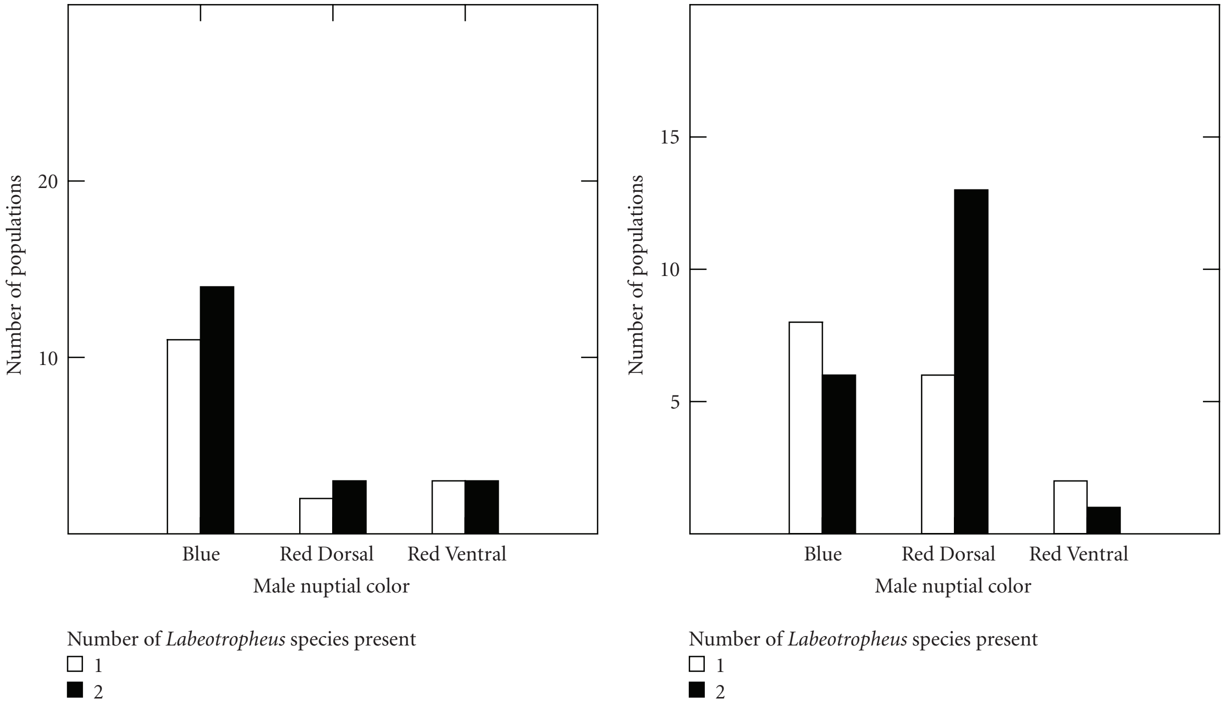


Figure 3: Predictive relationships between environmental variables and *Labeotropheus* color patterns. (a) Frequency of *Labeotropheus* species at each habitat type. (b) Maximum depths at which each *Labeotropheus* body color type is found. (c) Maximum depths at which *Labeotropheus* nuptial color types are found.

This finding suggests that male nuptial coloration is a signal of specific status in the *Labeotropheus* and is thus probably an important component of the reproductive isolating mechanisms or mate-recognition systems between sympatric pairs of *Labeotropheus*. Further, male mbuna do not tolerate similarly colored opponents in their territories [17], so the different male coloration between the *Labeotropheus* species at locations where they are sympatric could also be the result of intrasexual selection.

The results presented herein strongly suggest that the nuptial color patterns of male *Labeotropheus* may be the result of naturally and sexually selected constraints. This



(a)

(b)

Figure 4: Frequency of nuptial color types at sympatric and allopatric populations of *Labeotropheus*. (a) *L. fuelleborni*. (b) *L. trewavasae*. While the proportions of nuptial colors displayed by *L. fuelleborni* remain relatively consistent whether found with or without *L. trewavasae*, note the marked increase of Red Dorsal coloration in populations of *L. trewavasae* sympatric with *L. fuelleborni*.

Table 5: Standardized between-set regression coefficients.

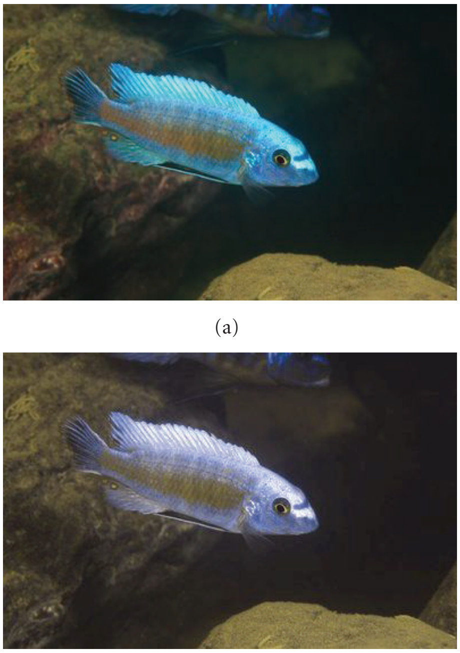
	Species (1)	Nuptial Color (1)	Nuptial Color (2)	Body Color (1)
Latitude	−0.021	0.107	−0.346	−0.409***
Longitude	−0.049	−0.122	−0.061	−0.030
Minimum depth	−0.345***	−0.341	0.001	−0.071
Maximum depth	0.395**	0.661*	−0.479	0.415**
Modal depth	0.819***	−0.151	0.294	−0.281
Habitat (1)	−0.006	−0.040	−0.119	−0.001
Habitat (2)	−0.329***	−0.027	−0.115	−0.136
Number of *Labeotropheus* species	0.109	0.243*	−0.129	0.010

$^{*}P \leq .05$; $^{**}P \leq .01$; $^{***}P \leq .001$.

conclusion, while in definite need of further confirmation and refinement through gathering new field and laboratory data, serves to underline the equivocal nature of the results of similar studies. Thus, as Dalton et al. [25] suggest, putative associations between male nuptial coloration and environmental variables in the haplochromine cichlids deserve much more attention, especially since much has been written regarding the importance of male color pattern and speciation in these fishes. As such, exacting measurements of the spectral environment made at both shallow and deep-dwelling populations of *Labeotropheus* would aid in understanding the conditions in which these signals and receivers coevolved. Further, while laboratory studies have confirmed that male coloration is important in sexual selection, *in situ* studies of natural mate choice tendencies or experimental manipulations of inter- and intrasexual encounters under natural lighting conditions could be very useful in further untangling the roles of natural and sexual selection in the evolution of male coloration.

4.1. A Postscript: Why Red? Why Not "One Fish, Two Fish, Blue Fish…Bluer Fish"?

Red or other carotenoid-based colors present a number of challenges for fishes. In many cases, such pigments are derived from dietary sources, necessitating a search for the foods that will yield the dietary precursors to synthesize these pigments [46]. The attenuation of long-wavelength light with depth presents an additional challenge; if a fish wants to be cryptic or to have a "private," short-range signal, red



(a)

(b)

Figure 5: A comparison between the appearance of a predominantly carotenoid-based *Labeotropheus* color pattern as seen by a trichromatic human (a) and a protanopic human (b). While largely heuristic, this figure illustrates that even without a long-wavelength-sensitive photoreceptor, a protanopic human or mbuna (like a *Labeotropheus*) could still perceive the contrast between the carotenoid patch at midbody and the rest of the fish. It should be noted that Figure 4(b) does not take into account either the ultraviolet-sensitive photoreceptor of the *Labeotropheus* or the attenuation of red and orange light at the depths at which this fish, a *L. trewavasae* from Manda, Tanzania, would typically be found. The original photo (a) is generously provided by Ad Konings/Cichlid Press, and the Daltonized photo (b) was produced from the original using the Vischeck algorithm, available at http://www.vischeck.com.

coloration might be a good option. But what about fish that visually search for mates and for whom nuptial color signals are of extraordinary importance in identifying mates or even competitors (e.g., [17])? Further, for fish like many of the mbuna, and the *Labeotropheus* in particular, that lack a long-wavelength-sensitive photoreceptor [25, 47], the use of carotenoid-based pigments is even more puzzling; why utilize pigments to which your visual system is insensitive? The answer most likely has to do with within-pattern contrast.

Pauers et al. [16] demonstrated that females of *L.* c.f. *fuelleborni* "Katale" consistently preferred males with the highest degree of contrast among the various color pattern elements present on the flanks of the fish. The males of this population are an example of a Red Ventral nuptial color pattern, with a prominent orange patch on their sides below their lateral line; in some individuals, this patch might comprise more than 67% of the surface area of their flanks. Further, Dalton et al. [25] found that many of the mbuna species they examined also had a high degree of contrast within their color patterns, including species that, like the *Labeotropheus*, lack a long-wavelength-sensitive photoreceptor. While anthropomorphism is always dangerous, these fish may see the world, in part, like a protanopic human, who also lacks a long-wavelength-sensitive photoreceptor, but sees reds as a different color entirely (Figure 5); indeed, the inability to discriminate amongst red and reddish hues may be the reason why there are so few examples of true, saturated reds in the *Labeotropheus* and other mbuna. Nonetheless, as long as an observing fish's percept of the carotenoid pigment patch contrasts with the percept of the neighboring patch or patches, the overall integrity of the signal is maintained, despite the lack of sensitivity to long-wavelength colors.

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Research Article

Repeatability and Heritability of Behavioural Types in a Social Cichlid

Noémie Chervet, Markus Zöttl, Roger Schürch, Michael Taborsky, and Dik Heg

Department of Behavioural Ecology, Institute of Ecology and Evolution, University of Bern, 3032 Hinterkappelen, Switzerland

Correspondence should be addressed to Dik Heg, dik.heg@iee.unibe.ch

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Aim. The quantitative genetics underlying correlated behavioural traits ("animal personality") have hitherto been studied mainly in domesticated animals. Here we report the repeatability (R) and heritability (h^2) of behavioural types in the highly social cichlid fish *Neolamprologus pulcher*. *Methods*. We tested 1779 individuals repeatedly and calculated the h^2 of behavioural types by variance components estimation (GLMM REML), using 1327 offspring from 162 broods from 74 pairs. *Results*. Repeatability of behavioural types was significant and considerable (0.546), but declined from 0.83 between tests conducted on the same day, to 0.19 on tests conducted up to 1201 days apart. All h^2 estimates were significant but low (e.g., pair identity $h^2 = 0.15 \pm 0.03$ SE). Additionally, we found significant variation between broods nested within the parent(s), but these were not related to several environmental factors tested. *Conclusions*. We conclude that despite a considerable R, h^2 in this cichlid species is low, and variability in behavioural type appears to be strongly affected by other (non)genetic effects.

1. Introduction

Individuals within animal populations often differ consistently in how they cope with environmental and social challenges, for instance with some individuals typically reacting shy and nonaggressively to such (novel) challenges and others reacting bold and aggressively [1–3]. Often, these individual differences are referred to as "behavioural types" or "coping styles" (e.g., shy, bold), and behavioural traits may covary amongst each other (e.g., shy individuals are also nonaggressive and nonexplorative, whereas bold individuals are more aggressive and explorative, e.g., [4]). Correlated behavioural trait values on the population level are commonly denoted as animal personalities, behavioural syndromes, or temperaments [5]. Central questions in animal personality research are (i) whether differences in behavioural types have a genetic basis, that is, whether they are heritable; (ii) whether and to what extent individuals remain consistent in their behavioural traits over time, that is, whether behavioural responses of individuals are repeatable [6–8].

The genetic components affecting the expression of different personalities have been well explored in humans (see reviews e.g., [9–15]), domesticated animals [16, 17], and animal model systems [18–20]. The evolutionary ecological factors responsible for the evolution of variation in behavioural types remains somewhat enigmatic and difficult to explain in natural animal populations, despite recent theoretical advances showing how life-history tradeoffs might generate and maintain such variation [21, 22]. In principle, consistent individual differences and covariation in behavioural traits are a paradox in evolutionary biology, particularly if such differences have a genetic basis. Standard theory would expect each individual to flexibly adjust their behaviour to the environment. For instance, in a predator rich environment each individual should devalue future fitness in favour of current fitness and adjust their behaviour accordingly (e.g., hide more and be less explorative).

In the human psychology literature it has been acknowledged that although personality differences have a genetic basis [9–15], other (social) factors might impinge upon and alter the expression of human behavioural types over a lifetime, including for instance life events (like the death of a partner, [23]). If personalities are truly fixed over life and can be accurately measured by using standardized questionnaires (e.g., [24]), or by using standardized observational assessments (e.g., [25–28]), or behavioural tests (as used to determine personalities in many invertebrates and vertebrates, see [2]), the repeatability of behavioural types should approach one. Clearly, this is not the case in humans ([29]; where it increases from 0.31 in childhood to a plateau of 0.73 beyond 50 years of age: [30]) and in most animal studies [7], which led to the proposition to incorporate behavioural reaction norms into the animal personality concept [6]. By incorporating behavioural reaction norms, animal behaviour can be analysed using standard game theory, where behavioural strategies may be governed by internal "state" (e.g., body condition, sex, status, and reproductive activity), sometimes resulting in alternative strategies (e.g., depending on morphology or age), but where these strategies and behaviours are not necessarily fixed for life (i.e., without the need to assume the existence of animal personalities). In fact, this has been the major criticism of animal personality research: if the long-term stability ("repeatability", R) is not proven in any study population, why not assume that the current variability between individuals reflects the current variability in "states" of the individuals tested? Using a meta-analysis, Bell and others showed that the repeatability estimate significantly declined with the time interval between the different tests [7]. As the number of long-term repeatability studies in animal personality research is still very low [31] and biased towards domesticated animals [32–42], our first target is to test for the long-term repeatability of behavioural types, using descendants of a wild population of a cooperatively breeding cichlid, *Neolamprologus pulcher*.

We use the cichlid *N. pulcher* as model species, where individuals have been shown to differ consistently in behavioural traits (across the bold-shy continuum) in both the field [43] and the laboratory (stocks derived from the same field population, [4, 39, 44–46]). In addition, males and females have been shown to remain relatively stable in trait values from the juvenile stage (when they are the small, subordinate helpers of an adult pair) to early adulthood (when they are large, subordinate helpers and reproductively mature [39]). Behavioural types in this species may also influence sociality, reproduction [46], and helping behaviour [39, 44, 47]; and also alloparental brood care differs consistently between female helpers [47]. The major hypothesis proposed to explain this variability in behavioural types suggests that subordinates trade-off effort to gain social dominance inside versus outside their territory, which either selects for distinct life-history strategies (e.g., nonexplorative, helpful, and risk-aversive individuals opt for dominance inside their group's territory, whereas explorative, bold, and aggressive individuals opt for dominance outside their group's territory, which involves early dispersal and independent breeding [39, 48]) or for diverse ontogenetic trajectories in behavioural types (e.g., young fish being risk aversive whereas older fish being risk prone). Here we expand the time frame of standardised tests to encompass the lifetime of these fish.

The second target of this study has been to estimate the genetic variance underlying phenotypic variation in personality traits in *N. pulcher* ("heritability" h^2 [49, 50]), and to compare estimates of R and h^2. Repeatability R often sets the upper limit to the heritability of a trait, and both measures are correlated in comparisons across species or populations, if the phenotypic variances in the compared units are governed by similar processes involving additive genetic variance. Any species or population showing a high heritability in a behavioural trait should also show a high repeatability in this trait (as genes are more likely to be involved in the determination of the behaviour). In contrast, a high repeatability can coincide with a low heritability, if the behavioural type is based, for instance, on the (current) internal state of individuals or on their (life-history) strategy [21] or social strategy [51], which may cause variation that is largely independent from genetic effects. Maternal or paternal effects, maternal additive genetic effects and genotype × environment interactions may also yield discrepancies between the heritability and the repeatability estimates (e.g., with heritability exceeding the repeatability, [52]). Both low repeatability and low heritability would indicate that the population exhibits no animal personalities, particularly if the repeatability diminishes over time.

In *Neolamprologus pulcher*, personality traits (such as boldness, aggressiveness, and propensity to explore) are consistently different between individuals (see references above) and related to two major life-history decisions: whether to help and whether to disperse. Furthermore, these traits are not related to growth rate in fish kept singly [39], but if living in groups consisting of members with divergent personality types, shy fish were found to grow quicker in body length than bold fish [53]. Studies of personalities are particularly interesting in social species like cichlids and primates [54], as they may bear similarities to the human personality axes which incorporate significant aspects of human behaviour and sociality (e.g., "OCEAN": Openness, Conscientiousness, Extraversion, Agreeableness, but with the possible exception of Neuroticism [24, 55–58]). Human and animal personality differences may be governed by similar differences in (neuro)physiological responses to environmental challenges and stressful situations [23, 59–62]. In this study we first estimated the repeatability of behavioural types based on a combined score of boldness, aggressiveness, and exploration propensity by comparing test results of individuals obtained successively with intervals ranging from 0 to 1201 days (which approaches the maximum life span of this species: see [63]). In a second step we estimated the heritability of these same behavioural types using parent(s)-offspring regressions and variance component estimations from offspring derived from different broods of the same pair.

2. Methods

2.1. Study Species. Descendants of wild caught *Neolamprologus pulcher* (from wild animals collected in 1996, 2006, and 2009 near Kasalakawe, Zambia, so-called three different "stocks" and their crossings) were used for this study and tested in the years 2005–2008 and 2010. These fish are well-studied cooperative breeders [64], endemic to Lake

Tanganyika where they live in breeding groups composed usually of a dominant breeder male, one to several breeder females and some helpers [65]. All fish were fed twice a week with fresh food (JBL *Cyclops* spp., shrimp, *Artemia* spp., mosquito larvae) and the other five days with JBL De Novo Lake Tanganyika cichlid flake food (except for some missing days due to absence). This is the standard feeding regime for all cichlid fish at our laboratory. During 2010 we additionally fed all individuals on six to seven days per week with fresh small food items (*Artemia* freshly hatched eggs and JBL *Cyclops* spp., the latter replaced with *Daphnia* spp. when all offspring of all broods had grown beyond 10 mm standard length). This was done to ensure that all offspring received enough food to grow proficiently and to reach testing age. Cichlids were kept in tanks within climatised rooms (24–29°C, lights on between 08:00–21:00 h).

2.2. Experimental Setup. The experimental setup and tests performed in 2005 and 2006 were reported in [39] (repeated tests of the same 36 individuals up to 150 days apart by Roger Schürch), tests performed in 2007 were reported in [4] (repeated tests of the same 272 individuals up to 53 days apart by Susan Rothenberger). In 2008 (unpublished) repeated tests were performed of 32 individuals by Liana Lasut, Estée Bochud, and Sebastian Keller (using the same setup as [4]), and 10 individuals retested in 2010 by Noémie Chervet and Dik Heg). These data were used to calculate the repeatability of behavioural types for time intervals of up to 1201 days after the first test was done. Individuals were marked between 2005 and 2008 with colour marks or fin clips and kept in breeding pairs, or fitted with PIT transponders and kept in aggregation tanks up to the moment of re-testing in 2010.

In September 2009 new breeding pairs were established and allowed to breed without monitoring their clutches (all "control pairs"), their offspring were tested without any information about the clutches (and thus offspring were up to 6 months of age). Between March and August 2010 these pairs were augmented with more new breeding pairs that were allowed to breed as follows.

Control pairs ($n = 19$ pairs) were kept in 89 or 93 litre tanks (length × breadth × height cm, height water level cm: 60 × 40 × 40 cm, 37 or 50 × 50 × 40 cm, 37 cm; resp.) and one clutch was left to hatch inside their tank (usually the first clutch), and these offspring remained there until personality testing (Figure 1(a), clutch treatment "with parents", see below for more details). All other clutches were removed on the day of egg laying and put separately to hatch in a 24-litre tank (40 × 25 × 25 cm, 24 cm; clutch treatment "isolation", see below for details). As pairs may produce a clutch about every other week [66], removal of the clutches ensured that we knew the identity of the offspring if they remained with their parents. However, some clutches remained undetected and were discovered after hatching and in such cases we removed all the offspring to an isolation tank as soon as possible. If the parents had very large offspring in their tank from a previous brood, we allowed them to hatch and keep a second clutch, as the offspring from the two broods could be easily distinguished according to their large size difference (this occurred only in 2 pairs). In 4 pairs a pair member died when already offspring were present and these offspring were removed and stored in a separate tank until personality testing, and the dead partner was replaced with a new partner.

Cross-breeding pairs ($n = 38$ pairs that produced at least one clutch, includes 17 pairs from [46]) were kept in 54 or 58 litre tanks (60 × 30 × 33 cm, 30 cm; or 60 × 30 × 35 cm, 32 cm). However, they included repeated measures of the same female with different males, and vice versa, so in total 19 different females and 20 different males were involved. The pairs were left together to breed for one and a half months. In between they were remeasured and reallocated to different mates. In total, we attempted to mate each individual with 5 different mates, but were only successful (i.e., the pair produced at least one clutch in 1.5 months) for a maximum of 4 different mates (females: 7 × 1, 8 × 2, 1 × 3 and 3 × 4 mates; males: 7 × 1, 9 × 2, 3 × 3 and 1 × 4 mates), partly because we lost and had to replace 4 individuals intermittently. All clutches were removed into 24-litre tanks (Figure 1(a), clutch treatment "isolation"), penultimate clutches were used for the clutch treatment "with parents" (in their 54 or 58 litre tanks) or "with foster parents" (see below), to increase the sample sizes for these latter two treatments.

Cross-fostering pairs ($n = 14$ pairs that produced at least one clutch, in one pair their single clutch did not hatch) were kept in 54 or 58 litre tanks (60 × 30 × 33 cm, 30 cm; or 60 × 30 × 35 cm, 32 cm). Their first clutch was removed into a different empty 54 or 58 litre tank (Figure 1(a), clutch treatment "cross-fostering", see below for details), however, if this clutch did not hatch, the second clutch received the same treatment. All other clutches were removed into the treatment "isolation" (24 litre tank). Pairs kept producing clutches until they were transferred as foster parents to a foster clutch (see Clutch treatments below).

2.3. Clutch Treatments. All pairs were checked every day for new broods. Upon detection of a new clutch, we commenced with a 15 min brood care observation, counting the frequency of cleaning the eggs (each mouth movement counted) and fanning the eggs (aerating the eggs by vibrating with body and fins [67]) for the female and the male separately [47, 66, 68]. The minimum distance to the eggs in cm (with 0 indicating inside the pot(s) containing the eggs) was also noted for the female and male separately. Unfortunately, pairs could also lay clutches under/behind the filter or on the aquarium walls, and these clutches were sometimes not detected until the fry hatched (which occurs 2 to 3 days after egg laying). These cases account for some missing data on clutch size, average egg mass, hatching success (but not the number of hatched offspring, as fry were immediately counted), and brood care behaviour. After the brood care observation, the pot was removed and we counted the number of eggs (clutch size) and measured the average dry egg mass (by sampling up to 5 eggs per clutch and weighing them after 32 h drying in a 70°C oven). Data on brood care, clutch size, and egg mass will be treated in detail elsewhere.

The broods in the treatment "with parents" broods were then immediately placed back with their parents

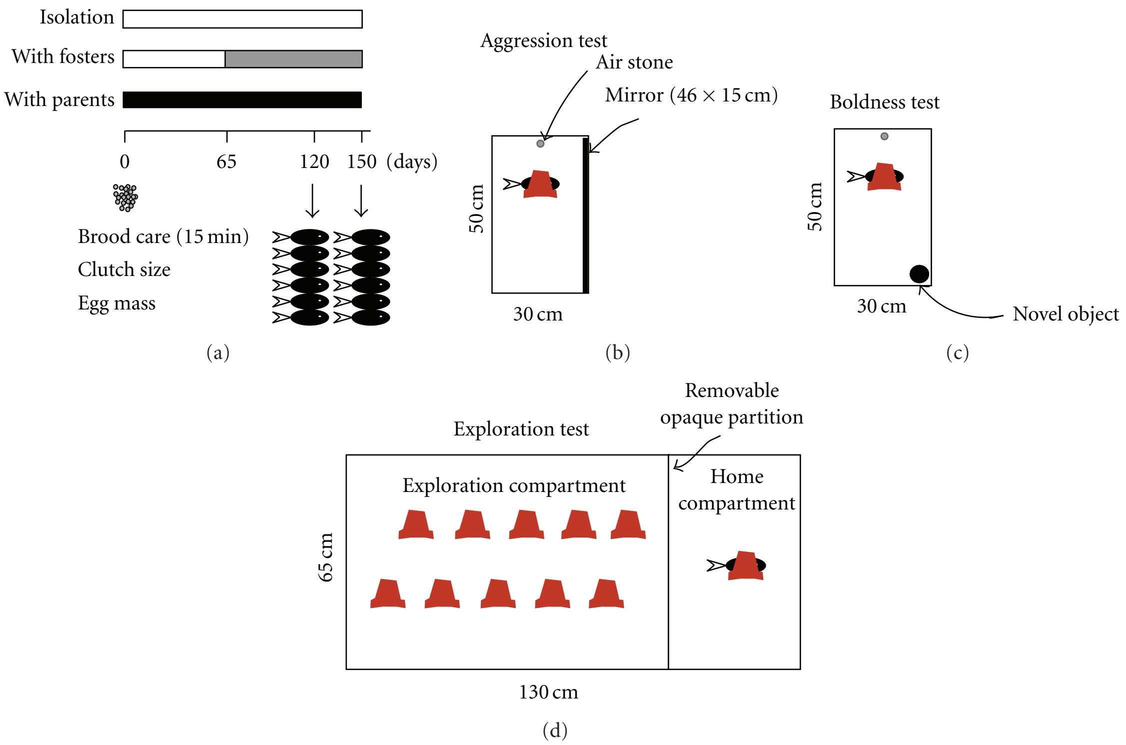


Figure 1: Treatment of the broods and experimental setup of the three behavioural tests (black fish show the starting position of the focal individual in each test). (a) Offspring remained with their parents (treatment "with parents"); or were isolated and raised only together with their siblings (treatment "isolation"); or were isolated, raised together with their siblings for 65 days, and from this day onwards received a foster pair ("with fosters"). Six offspring were removed for behavioural testing on days 120 and 150 each (or fewer offspring if less than 6 offspring were still alive on day 120, and fewer offspring if less than 6 not yet tested offspring were still alive on day 150). Offspring were measured and moved singly to a 40-litre tank depicted in (b, c). After two days acclimatization, each offspring was tested. Note that offspring were permanently removed to avoid confusion with previously tested offspring. (b) Setup of the aggression test, where aggressive displays/attacks were scored towards the mirror (either placed left or right), and hiding time inside the pot was measured. (c) Setup of the boldness test, where latency and shortest distance moved to the novel object was scored (object placed left or right). (d) Setup of the exploration test, the test started by removing the opaque partition, and latencies plus visits to 10 pots were measured. Note that the home compartment was either on the left or the right and pots were shifted accordingly, visits to the home compartment pot were not counted. Offspring and parents were similarly tested according to (b–d).

(Figure 1(a)). The broods in the treatments "isolation" and "with foster-pair" broods were permanently removed (Figure 1(a), the pair received a new clean flower-pot halve) and placed into an isolation net inside a separate 24-litre tank ("isolation") or 54/58 litre tank ("with foster-pair"), and the eggs were incubated using an air stone. Approximately five days after hatching they were released from their isolation net. "Isolation" broods were kept in their 24-litre tanks with their siblings until 55 to 114 days after egg laying, when they were transferred to a bigger tank (34 to 188 litre) to accommodate both their size and numbers (as numbers were highly variable at transfer, ranging between 2 and 67 siblings, we also used highly variable tank sizes). Two broods with a single offspring each and three broods with two offspring each remained in their original tanks, as transfer was not necessary due to their low number and limitations in the availability of bigger tanks. "With fosters" brood were kept together with their siblings in their 54/58-litre tank, until they received a foster pair from day 65 onwards (Figure 1(a)).

2.4. Body Measurements and Personality Testing. Before the personality test (on day −2 before testing) or mate exchange (on day 0 of release with the new mate), each individual was sexed (external papilla inspection under a dissecting microscope), measured (standard length SL and total length TL in 0.1 mm using a dissecting microscope) and weighed (body mass in mg), and fin clipped for a DNA sample and for male/female identification within pairs. All offspring produced in the period September 2009 to March 2010 were tested in 2010, their parents were tested in March 2010 (5 females had lost their mate before March 2010, so 5 pairs had missing data for the male parent; 1 male had lost his mate before March 2010, so 1 pair had missing data for the female parent). The reasoning for testing all offspring was that they had experienced a very prolonged time with their parents, so might be well suited to serve as a benchmark for future studies. Offspring produced in the period March to August 2010 were identified by their clutch identifier and clutch treatment and tested on day 120 since the clutch was laid

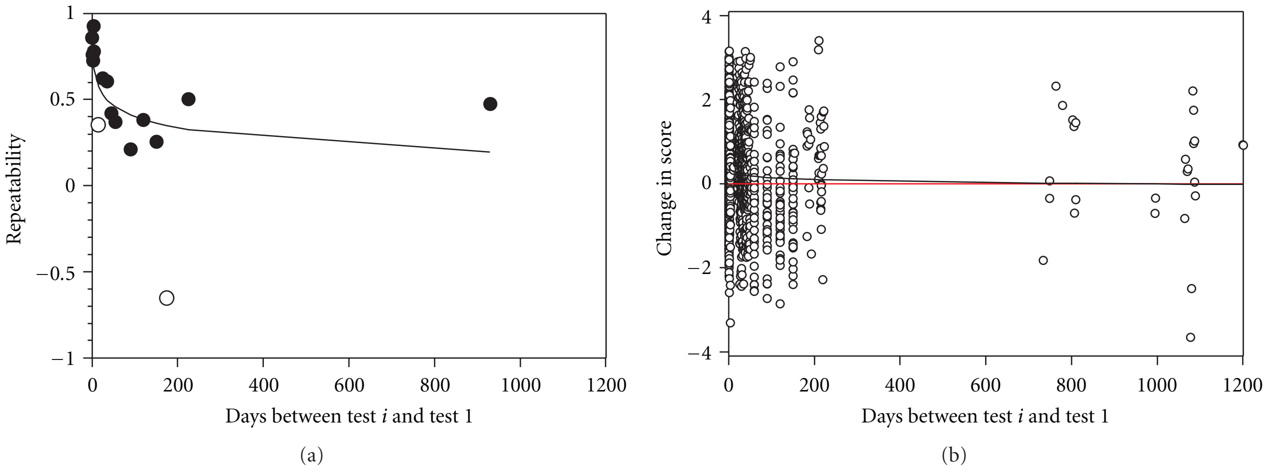
(Figure 1(a): the six largest siblings, or less if less were alive). An additional sample of the next 6 largest siblings was taken for tests on day 150 after clutch production for all "with parents" treatments, "with foster-parents" treatments, and the first clutch of the "isolation" treatment (Figure 1(a)). We were not able to take additional samples for all second and later clutches of the "isolation" treatments due to time constraints.

The personality testing procedure has been outlined in detail in [4, 39]. Briefly, boldness and aggressiveness tests were conducted for each single focal fish inside a 42-litre tank (Figures 1(b) and 1(c), 50 × 30 × 30 cm; 28 cm water level). In total, 34 of such tanks were available for testing. Focal fish were left for two days to acclimate and settle territory around a single flower pot halve (placed 30 cm from the front glass). In the aggressiveness test, a mirror was placed along one side (Figure 1(b)). Here the total time hiding (in seconds) and aggressive behaviours towards the mirror image were noted: restrained aggression (frequency of slow approach, fast approach, head down display, spread fins, s-bending) and overt aggression (frequency of contact with the mirror, again 5 min total test duration). In the boldness test, a novel object (Figure 1(c), plastic beetle, plastic funnel, plastic blue half moon, clay bird, or plastic white cross) was placed in a front corner (left or right), and the latency to approach this object (in seconds) plus the closest distance to the object (in cm) was recorded (5 min total test duration). The exploration test was conducted in a 400-litre tank, where the fish were left in a partitioned area with a flower pot half for ten minutes before testing (Figure 1(d), so-called "home compartment"). Then the partition wall was removed and the focal fish could start exploring the unknown part of the tank where ten other flower pot halves were placed ("exploration compartment", Figure 1(d)). Here the time spent moving outside the pots in any compartment, the latency before leaving the home compartment (in seconds), and for the exploration compartment, the latency before entering the first pot, the number of pots approached, the number of pots entered, and the number of different pots entered (0–10) were recorded (again 5 min total test duration). The three tests (boldness, aggressiveness, and exploration propensity) were conducted in randomized order. The three tests were repeated one day later, or rarely on the same day or up to four days later (due to time constraints and tank constraints). Note that in 2005 and 2006 all three tests were conducted in the 400-litre tank and the exploration test lasted 10 min (instead of 5 min, observer Roger Schürch, see [39]). Moreover, due to severe time constraints, the exploration test could not be conducted for all focal animals in 2010, as it involved a lot of time lost in handling fish. See the description of statistical analyses for details on how we have dealt with these differences in procedures.

2.5. Statistical Analyses. We used Categorical Principal Components analyses CatPCA with two-knot spline transformations [69] to summarise the three different tests (boldness, aggressiveness, and exploration propensity) into a single measure of "behavioural type" (object scores, see also [4]). To account for the observer effects, each CatPCA was run separately for each observer (2005-2006: Roger Schürch $n = 216$ series of three tests, 2007: Susan Rothenberger $n = 1042$ series of three tests, 2008: Sebastian Keller/Estée Bochud/Liana Lasut $n = 64$ series of three tests, boldness/aggressiveness/exploration tests; 2010: Markus Zöttl $n = 288$ tests, including 264 tests where only aggressiveness was recorded; Noémie Chervet $n = 1412$ series of two tests and Dik Heg $n = 1268$ series of two tests: aggressiveness and boldness). Noémie Chervet and Dik Heg also conducted exploration tests, but these were excluded from the analyses to keep the data amongst the individuals consistent. This procedure has the advantage that first, all observers automatically scale to a mean "behavioural type" of zero; and second, the different test procedures are also scaled to a mean "behavioural type" of zero (i.e., in 2005-2006 all three tests were conducted inside a 400-litre tank and the exploration tests lasted 10 min versus in 2007–2010 the three tests were conducted according to Figures 1(b)–1(d) and always lasted 5 min). This procedure only assumes that all observers capture more or less the complete variation in behavioural types present in the population, which is a reasonable assumption considering the large number of tests each observer conducted, and that all original variables had very high correlations both before and after transformations in the CatPCA, for each observer separately.

Repeatability was estimated using the VARCOMP and RELIABILITY procedures in SPSS 17 [70], by extracting the variance components and the corresponding intraclass correlation coefficients (= repeatability) by using the Restricted Maximum Likelihood method (REML). The procedure was run once for the complete data set using VARCOMP (Restricted Maximum Likelihood Method REML) and once for each time difference between the first test and the next test(s) i, where individuals were tested up to 6 six different times using the RELIABILITY procedure (which was easier to use in the latter analyses for data management reasons). Time differences between test i and the first test was calculated in days and the repeatability was calculated for days = 0 (test i conducted on the same day as the first test), 1, 2, 3, 4, 15 (between 11 and 20 days), 25 (between 21 and 30 days), 35 (between 31 and 40 days), 45 (between 41 and 50 days), 55 (between 51 and 60 days), 90, 120, 150, 175 (between 151 and 200 days), 225 (between 201 and 250 days), and 930 days (between 732 and 1201 days). To estimate the change in repeatability over time, these 16 estimates of repeatability (from 0 to 732–1201 days) were regressed against ln (days + 1) weighted by their sample size (weighted linear regression). Similarly, change in the test scores (test i minus the first test) were analysed by regressing this difference against ln (days + 1).

Heritability was estimated using (1) the mid-parent versus mid-offspring weighted regression slope (weighted by the square root of the number of offspring tested [71]). (2) The intraclass correlation coefficients derived from the variance components extracted using the VARCOMP procedure in SPSS 17 for random effects of the pair identity, mother identity, and father identity, respectively, using the REML method. These estimates were verified by using the minimum norm unbiased estimator method, using both



(a)

(b)

Figure 2: Repeatability of behavioural types significantly declined over time. (a) Pairwise repeatability from the test results (behavioural type test *i* versus behavioural type first test), from two test series conducted on the same day 0 ($n = 44$), 1 ($n = 1435$), 2 ($n = 277$), 3 ($n = 18$), 4 ($n = 45$), 11–20 ($n = 7$),21–30 ($n = 101$), 31–40 ($n = 101$), 41–50 ($n = 63$), 51–60 ($n = 48$), 90 ($n = 36$), 120 ($n = 36$), 150 ($n = 36$), 151–200 days ($n = 7$), 201–250 days ($n = 15$) and 732–1201 days apart ($n = 18$), respectively. The two low sample sizes of 7 are indicated with white circles, but do not affect the regression line, as the line was fitted weighing by the sample size. (b) Pairwise changes in the test series results over time (behavioural type test *i* minus behavioural type first test, $n = 2501$). Note the maximum time difference between two tests of 1201 days. See the text for the regression line.

the priors 0 or 1 (MINQUE(0) or MINQUE(1) method: see [70]), and since the MINQUE estimates were virtually identical to the REML estimates only the latter are given. Finally, fixed effects on the brood level were tested by General Linear Mixed Models, using as random effects pair identity and brood identity nested within pairs (and extracting the variance components accordingly). The following fixed effects were tested: female stock (1996, 2006, 2009), male stock (1996, 2006, 2009), treatment of the brood (with parents, with foster parents, isolated), volume of the tank in litres (both before and after transfer, if offspring were not transferred volumes were identical), temperature of the tank in degrees Celcius (both before and—if this applies after transfer), and body size of the focal offspring (SL mm). Note that the fixed effects were measured on the brood identity level, and therefore varied between broods within pairs, and that the offspring varied in body sizes (both within and between broods).

Due to replacement of dead mates, we ended with a total sample size of 74 pairs, 162 broods, and 1327 offspring tested for the heritability analyses (from 49 individual females and 50 individual males), in one pair the mother was not tested and in three pairs the father was not tested.

3. Results

3.1. Categorical Principal Component Extraction of Behavioural Types. In total 1779 individuals were tested two to six times for their behavioural traits (on average 2.41 tests per individual or 4290 tests in total). Categorical Principal Components analyses were run for each observer separately, and in each case a single factor was extracted with an Eigenvalue higher than 1 explaining a high proportion of the correlated behaviours in the one to three tests conducted (Table 1). The extracted factor scores were saved as the "behavioural type" of the individual in each test (for the repeatability analyses) or averaged per individual over all their tests as "behavioural type" (for the heritability analyses).

3.2. Repeatability of the Behavioural Types. VARCOMP analysis (REML) showed significant repeatabilities of the behavioural types ($n = 4290$ tests of 1779 individuals): the variance attributed to the individuals was 0.5495, the error variance was 0.4576, which gives a repeatability (intraclass correlation coefficient) of $0.5495/(0.5495 + 0.4576) = 0.5457$ (± 0.0149 SE, standard error of the estimate). However, by comparing the test results pairwise with the first test result over time (from 0 days between two tests up to 732–1201 days between two tests) it became clear that the repeatability R significantly declined over time (Figure 2(a); regression analysis weighted by sample size: $R = 0.830 \pm 0.002 - 0.093 \pm 0.001 \times \ln[\text{days between tests} + 1]$; $F = 5864.7$, $P < .001$,$R^2 = 0.72$, $n = 16$ pairwise R estimates, ± SE). Both the intercept and the slope of this regression line (depicted in Figure 2(a)) were significantly different from zero ($t = 340.1$ and -76.6, resp., both $P < .001$).

Although the repeatability changed over time, the actual behavioural type test scores changed very little over time (Figure 2(b)). On a short-term basis, individuals became bolder, more aggressive, and explorative compared to their first test score, but this difference to the first test score rapidly diminished over time and approached the "no difference" (marked by the red line in Figure 2(b): $y = 0$). These changes were modeled with a regression analysis (black line in Figure 2(b): change in score = $0.442 \pm 0.027 - 0.065 \pm 0.012 \times \ln[\text{days between tests} + 1]$; $F = 29.1$, $P < .001$,

Table 1: Categorical Principal Component results for the behavioural testing, for each observer separately (in brackets the year(s) when the tests were conducted). The variance accounted for is represented by Cronbach's alpha, and the Eigenvalue is given (% explained variance in brackets). In each case a single factor score was extracted, and used to characterize the behavioural type of the focal individuals on a testing day (one test series), used for the repeatability analyses. Scores were then averaged per individual for the heritability analyses (scores from two to six test series averaged). Tests used for Categorical Principal Component extraction: B = boldness, A = aggression, E = exploration.

	n test series	Tests used per series	Cronbach's alpha	Eigenvalue
R. Schürch (2005-2006)	216	B, A, E	0.91	4.95 (61.9%)
S. Rothenberger (2007)	1042[a]	B, A, E	0.94	6.65 (60.5%)
E. Bochud/S. Keller/L. Lasut (2008)	64	B, A, E	0.94	6.93 (63.0%)
M. Zöttl (2010)	288[b]	A	0.85	2.30 (76.7%)
N. Chervet (2010)	1412	B, A	0.90	3.53 (70.4%)
D. Heg (2010)	1268[c]	B, A	0.89	3.49 (69.7%)

[a] Includes 72 individuals that were subjected to an additional test series following the procedure of Schürch and Heg [39] in the 400-litre tank, analysed separately with CatPCA: Cronbach's alpha = 0.93, Eigenvalue = 6.48 (58.9%).

[b] Includes 64 individuals for which also the boldness test was conducted, analysed separately with CatPCA: Cronbach's alpha = 0.92, Eigenvalue = 3.75 (75.0%).

[c] Includes 8 test series were only aggressiveness was scored and analysed separately with CatPCA Cronbach's alpha = 0.86, Eigenvalue = 2.36 (78.6%).

Table 2: Descriptive statistics of the offspring tested.

Parameter	n	Mean	SD	Minimum	Maximum
Treatment of the brood	1327	n with parents: 419, with foster parent: 91, isolation: 817			
Mother stock	1327	n 1996: 1031, 2006: 199, 2009: 97			
Father stock	1327	n 1996: 1166, 2006: 64, 2009: 97			
Tank volume litre[a]	1327	47.87	28.63	24[d]	93
Tank volume litre[b]	1327	88.45	48.13	24[d]	188
Tank temperature[a]	1219	27.17	1.22	24.25	29.50
Tank temperature[b]	1219	26.58	0.87	24.25	28.30
Offspring body size SL (mm)[c]	1327	24.11	10.27	7.0	77.0
Offspring behavioural type[e]	1327	−0.01565	0.87857	−2.20393	1.49879
n Offspring without missing values	1219				

Volume and water temperature of the offspring raising tanks: [a]before and [b]after transfer.

[c] Body size at personality testing.

[d] All clutches were produced by pairs in 54-to 93-litre tanks, but eggs and offspring from the treatment "isolation" were incubated and raised from the day of egg laying onwards inside 24 litre tanks and later transferred to larger tanks (see information given on tank volumes after transfer).

[e] Offspring behavioural type computed as the average of the average score per offspring—behavioural type-from the Categorical Principal Component analysis. Note that each offspring was scored at least twice (at least two test series of boldness test, exploration test and aggression test).

$R^2 = 0.012$, $n = 2501$, ± SE of the estimates, both the intercept and the slope of this regression line were significantly different from zero: $t = 16.3$ and -5.4, resp., both $P < .001$).

3.3. Heritability of Behavioural Types. The raw data of the offspring behavioural type scores are given in Figure 3 and descriptive data are provided in Table 2. First, we estimated heritability using weighted regression equations (weighing the regression analysis by offspring number; Figure 4, Table 2): that is, the mid-parent versus mid-offspring behavioural types (Figure 4(a)), mother versus mid-offspring behavioural types (Figure 4(b)), and father versus mid-offspring behavioural types (Figure 4(c)). Heritabilities were significant but low for the mid-parent and mother versus offspring regressions, and nonsignificant for the father-offspring regression (Table 3). Moreover, the heritability estimated from sibling comparisons was high and significant (last row in Table 3).

We should like to point out that the regression approach is inferior to the variance components method: first, because the regression approach assumes the behavioural type of the parents is measured without error; second, because the mixed nature of the data can be better accommodated by the variance components (using the REML method) and this method can be extended to estimate fixed effects (GLMM REML method, see below); third, because the above analyses suggest strong brood identity effects (last row of Table 3), which should be estimated as a random effect nested within pair identity effects (again using the GLMM REML method). We therefore recalculated heritability using the GLMM REML, once with the pair identity or parent's identity (female or male) as random effects (first three rows in Table 4) and once by adding also a brood identity nested within pair identity as random effects (last three rows in Table 4). Heritability estimates were all significant and now slightly larger than the previous estimates (cf. Table 4 with Table 3).

Table 3: Heritabilities h^2 of offspring behavioural type using the weighted regression equation approach for parents versus offspring (weighing the mid-offspring behavioural type by the square root of the number of offspring tested) and the one-way ANOVA approach for siblings (with brood identifier as a random factor).

Comparison	*n* pairs or parent, *n* offspring	MS	*df*, error *df*	*F*	Estimates Intercept ± SE	 Slope ± SE	h^2 ± SE
Midparent-offspring	70, 1272	0.95	1, 276	8.58**	−0.010 ± 0.024 ns	0.117 ± 0.040**	0.12 ± 0.04
Mother-offspring	49, 1318	2.10	1, 283	12.46***	0.008 ± 0.025 ns	0.095 ± 0.027***	0.19 ± 0.03
Father-offspring	50, 1281	0.07	1, 279	0.44 ns	−0.007 ± 0.025 ns	−0.019 ± 0.028 ns	0.00 ± 0.03
Siblings	162[a], 1327	1.90	161, 1165	3.08***	−0.004 ± 0.040 ns		0.41 ± 0.03

[a] Number of broods.

ns: nonsignificant, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

In total 74 pairs with 162 broods were tested (in one pair the mother was untested for behavioural type and in three pairs the father was untested for behavioural type). MS: mean square.

Heritability estimates are twice the slopes for mother-offspring and father-offspring regressions, and twice the intraclass correlation coefficient for siblings.

Table 4: Heritabilities h^2 of offspring behavioural type using the variance components approach (GLMM REML).

Random effect(s)	*n* pairs or parent, *n* offspring	Variance pair or parent	Variance brood	Variance error	Asymptotic covariance matrix[a] Within pair or parent	 Within error	 Between pair or parent versus error	h^2 ± SE
Pair	74, 1327	0.117***		0.653***	0.000787	0.000677	−0.000046	0.1524 ± 0.0316
Mother	49, 1318	0.121***		0.665***	0.001043	0.000697	−0.000035	0.3082 ± 0.0347
Father	50, 1281	0.097***		0.664***	0.000724	0.000716	−0.000036	0.2560 ± 0.0308
Pair + Brood within pair[a]	74, 1327	0.091**	0.069**	0.614***	0.000935	0.000641	−0.000002	0.1182 ± 0.0380
Mother + Brood within mother[a]	49, 1318	0.096**	0.074***	0.616***	0.001099	0.000649	−0.000007	0.2453 ± 0.0405
Father + Brood within father[a]	50, 1281	0.076*	0.085***	0.605***	0.000871	0.000647	−0.0000002	0.1980 ± 0.0387

[a] For brevity, covariances involving the random brood effects are not given ($n = 162$ broods for 74 pairs, $n = 160$ broods for 49 mothers, $n = 156$ broods for 50 fathers).

$^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

Heritability estimates are twice the intraclass correlations for mother and father effects, standard errors calculated according to [70].

Interestingly, brood identity random effects remained significant when nested within their parent(s) (last three rows in Table 4). This suggests siblings from the same brood shared a common (environmental) effect on their behavioural type. To explore potential shared effects, we added single fixed effects to a base model containing random effects of pair identity and brood identity nested within pair (Table 5). However, none of these effects significantly affected the behavioural types of the siblings: treatment of the brood, stocks of their parents, tank volumes and water temperatures during raising, and also offspring body size at testing were all clearly nonsignificant (Table 5).

4. Discussion

There are three main results. First, repeatability of behavioural type significantly declined over time, that is, for two tests conducted on the same day repeatability was 0.83, and for two tests conducted up to 1201 days apart repeatability was only 0.19. This time period spans the entire expected maximum life-span of this species, which has been estimated to be ca. 1000 days [63]. Second, heritability was low but significant and depended on the random effect fitted (i.e., pair, mother, father, or brood identity). Third, a significant random effect of brood identity within pair identity suggests a shared effect on the behavioural types of the broods, which did not depend on (i) the treatment of the broods, (ii) origins of female and male stocks, (iii) volumes and temperatures of tanks used to raise the offspring, and (iv) sizes of the offspring at testing. These three main findings are discussed in more detail below.

Temporal and systematic changes in behavioural type have been reported for various animal populations and for humans (e.g., [20, 33, 37, 40, 72–80]), including our

TABLE 5: Fixed effects on offspring behavioural type using the variance components approach (GLMM REML). In the base model only random effects of pair identity and brood identity nested within pair identity were added. Fixed effects were then tested stepwise for entry into this base model. See Table 2 for offspring sample sizes. Treatments of the brood were "with parents", "with foster parents", or in "isolation". Stocks were from 1996, 2006 or 2009. Volumes, temperatures, and offspring size are continuous (covariate) effects.

Fixed effect	Statistics when entered into base model			
	df	Error *df*	*F*	*P*
Treatment of the brood	2	100.2	0.54	.59
Mother stock	2	72.8	0.21	.81
Father stock	2	65.5	0.18	.83
Tank volume (litre)[a]	1	123.7	0.25	.62
Tank volume (litre)[b]	1	121.3	0.02	.90
Tank temperature (°C)[a]	1	112.5	0.30	.58
Tank temperature (°C)[b]	1	112.8	0.21	.65
Offspring body size (SL mm)[c]	1	440.0	0.29	.59

Volume and water temperature of the offspring raising tanks: [a]before and [b]after transfer.
[c]Body size at personality testing.

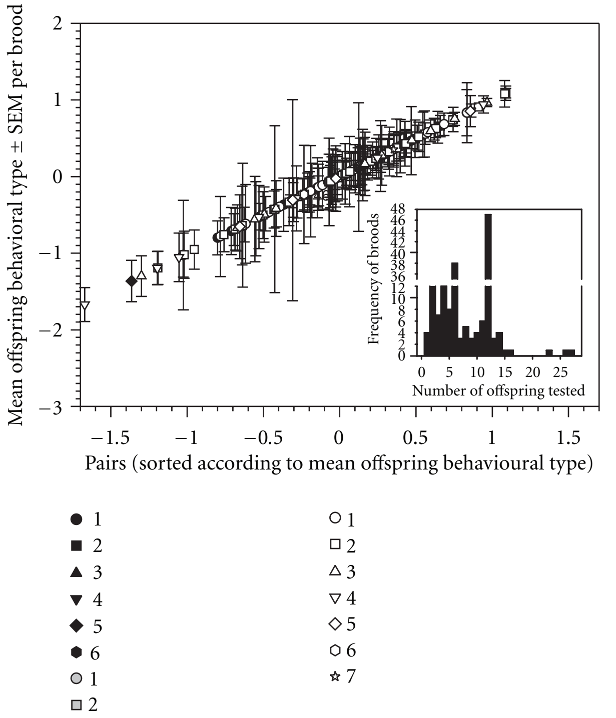
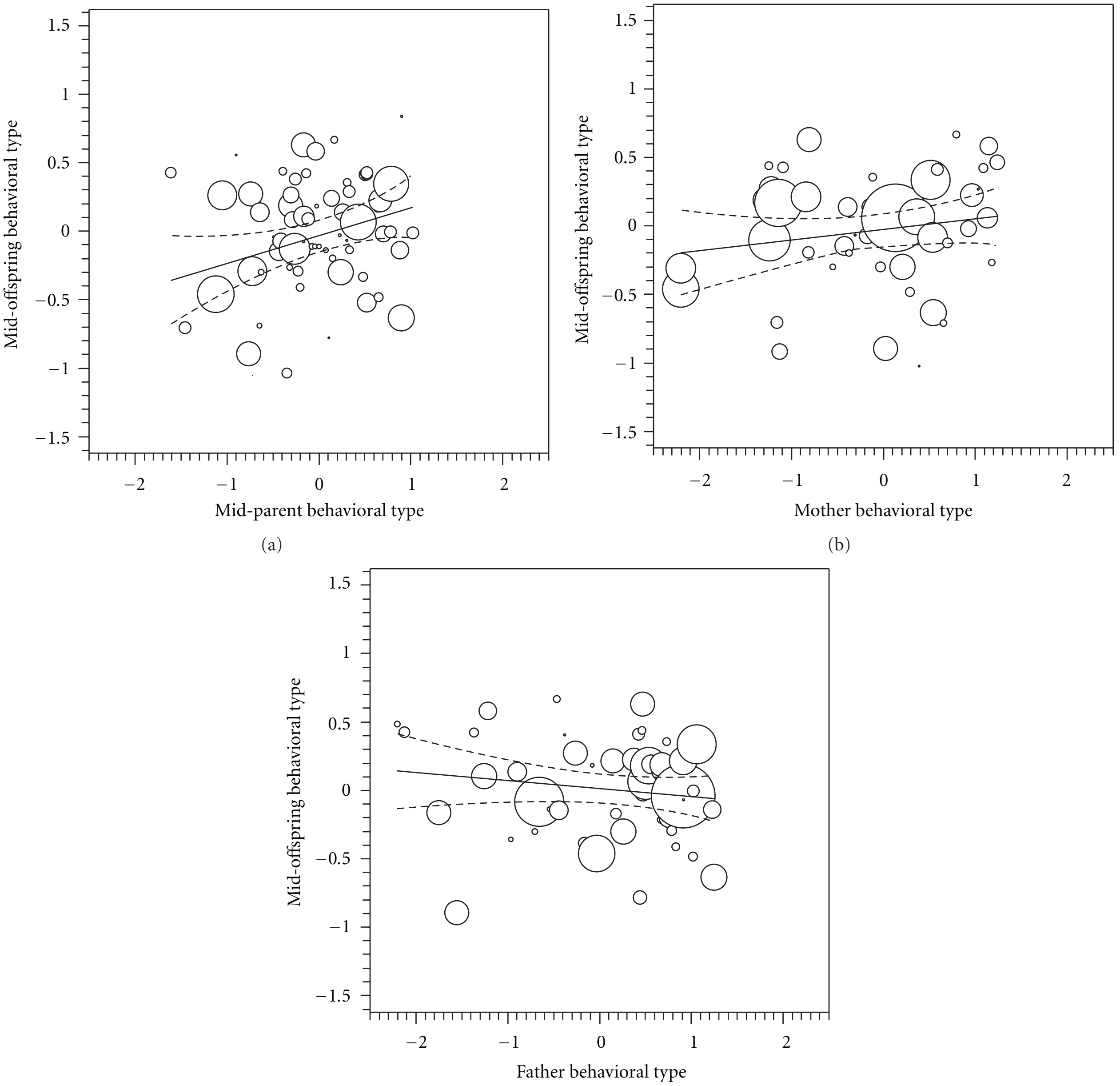


FIGURE 3: Mean offspring behavioural type per brood (±SEM, $n = 162$ broods), plotted per pair (pairs sorted by the mean of their offsprings behavioural types). Legend shows the brood treatments (black symbols: with parents, grey symbols: with foster parents, white symbols: in isolation) and the order of the brood produced (pairs produced up to 7 broods). Inset shows the number of offspring tested per brood ($n = 162$ broods with total 1327 offspring).

study species [39]. Although repeatability is a central role in animal (and human) personality research [7], it remains understudied. Clearly, if the repeatability is very low and also fluctuates or changes systematically over time, this would make the interpretation of individual differences in behavioural type liable to criticism and diminish the relevance of the underlying genetical effects. In our study, repeatability diminished strongly over time (Figure 2(a)), but nevertheless the behavioural type scores between two tests were quite comparable (Figure 2(b)). If two tests were conducted only a short time apart (e.g., on the same day), individuals were typically bolder, more aggressive, and explorative during the second test. This strongly suggests a training effect on the test scores of the individuals: a habituation effect which diminishes over time (see [36] for a similar example). Accordingly, if the two tests were conducted widely apart in time, test scores of individuals were on average very similar to each other. We note as a point of criticism that the change in repeatability over time was modeled by us using a simple regression model. However, it is quite likely (as the data in Figure 2(a) suggest) that the repeatability actually stabilizes to a level of 0.4 to 0.5 after 150 days between two tests. A "break-point regression" approach would be a way forward to study this, but would also need more repeatability data from day 150 onwards. We urge scientists to study the repeatability of behavioural types in more depth, as it plays a critical role in the concept of animal personality research, and we concur that these studies are particularly lacking in fish [39, 74, 75, 81–85].

We found that the heritability of behavioural type was ca. 0.15 in *N. pulcher*, which is a rather low estimate compared to other studies (see meta-analysis in [16]: mean = 0.31). However, heritability estimates may critically depend on the variance components which were actually tested, that is, whether the study design allowed testing for (permanent) environmental effects, maternal and paternal effects, and maternal additive genetic effects. Studies in domesticated animals have shown that many genetic and nongenetic factors may contribute to behavioural phenotype of the offspring (e.g., [86–91]). Similarly, we found strong evidence for shared sibling environmental effects (brood with pair effects in Table 3) and maternal/paternal effects on behavioural type (discrepancies between pair versus mother versus father effects in Table 3). This suggests that an animal



(a)

(b)

(c)

Figure 4: Heritability of offspring behavioural type using the regression approach. Symbol sizes represent the number of offspring tested per brood (pairs: 1 to 54, $n = 70$, excludes broods were one parent was not tested; mothers: 3 to 100, $n = 49$; fathers: 3 to 94, $n = 50$). Note that multiple broods tested from the same pair or parent, have the same x-axis value in each panel.

model statistical analysis of the behavioural types in *N. pulcher* might be a worthwhile enterprise in the future, to disentangle these variance components. We found no effects of some important aspects of the offspring's rearing environment on their behavioural type (like temperatures, tank sizes, and clutch treatments).

It is yet unknown how behavioural type (e.g., aggressive propensity) matches the behaviour shown under natural conditions (e.g., regarding dominance [92–95], territory acquisition [94, 96, 97], mate acquisition [59, 98–102], and mating performance [103]; for review see [85, 104]). It would be of particular interest to know how the behavioural type affects fitness and therefore is subject to natural selection. Field studies show that fitness effects of behavioural type may vary over time [105–107] and space [37, 94, 108–119], and behavioural type scores may not match one to one with actual behaviour shown in nature (e.g., due to context dependence, [120, 121]). Similarly, although exploration propensity is part of the behavioural syndrome in *N. pulcher* under laboratory conditions, it appears decoupled from the syndrome under natural conditions (i.e., actual distances moved in field settings: [43]). Under seminatural settings, shy fish have more socially positive interactions with their neighbourhood than bold fish, which is contrary to expectation [4]. However, as expected, bold fish are the hotspots of an aggressiveness network [4]. The relevance of all these effects for fitness in *N. pulcher* remain unclear, as (i) the effects of behavioural type are always small compared to other effects known to affect the behaviour and fitness of *N. pulcher* (e.g., social status, body size, and sex); (ii) frequencies of aggression, affiliation, and submission do not scale one to one on the behavioural type of the focal individual (Rothenberger et al. manuscript in preparation); (iii) effects of behavioural type on fitness (survival and reproduction) have not yet been measured in the field.

In domesticated and laboratory animals behavioural types (e.g., aggressiveness) are directly subjected to artificial selection by the experimenters [60, 122–136], for instance in order to reduce injury risk, fear, or anxiety in the animals, or when they serve as animal model systems. However, in natural populations it is yet unclear to which extent behavioural types are subject to natural phenotypic selection, or to which extent they are coselected with other traits under direct selection (e.g., age at maturity). Therefore, we consider it to be of prime importance for future studies to (i) map standardized behavioural test results (e.g., of aggressive propensity) on actual behaviour shown in nature under all relevant contexts (e.g., aggressiveness measured during all life-stages and types of contests), and (ii) obtain estimates about how different behavioural types relate directly (or indirectly) to fitness in the natural situation. Finally, our results suggest other (non)genetic effects affecting the behavioural type in *N. pulcher*, which should be analysed in more detail in the future.

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Research Article

Low Genetic and Morphometric Intraspecific Divergence in Peripheral *Copadichromis* Populations (Perciformes: Cichlidae) in the Lake Malawi Basin

Dieter Anseeuw,[1,2] Joost A. M. Raeymaekers,[3] Paul Busselen,[1]
Erik Verheyen,[4] and Jos Snoeks[3,5]

[1] *Interdisciplinary Research Centre, Katholieke Universiteit Leuven, Campus Kortrijk, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium*
[2] *KATHO, HIVB, Wilgenstraat 32, 8800 Roeselare, Belgium*
[3] *Laboratory of Animal Biodiversity and Systematics, Katholieke Universiteit Leuven, Charles Deberiotstraat 32, 3000 Leuven, Belgium*
[4] *Vertebrate Department, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, 1000 Brussels, Belgium*
[5] *Zoology Department, Royal Museum for Central Africa, Leuvensesteenweg 13, 3080 Tervuren, Belgium*

Correspondence should be addressed to Dieter Anseeuw, dieter.anseeuw@katho.be

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Peripheral isolated populations may undergo rapid divergence from the main population due to various factors such as a bottleneck or a founder effect followed by genetic drift or local selection pressures. Recent populations of two economically important *Copadichromis* species in Lake Malombe, a satellite lake of Lake Malawi, were neither genetically nor morphometrically distinct from their source populations in the main lake. Evidence was found for a founder effect which had a different impact on the genetic composition of the two species. In addition, the increased fishing pressure in Lake Malombe may have led to a reduction of the body sizes of both species.

1. Introduction

Peripheral populations are isolated or semi-isolated from the major distribution range of a species. The effects of geographical or ecological isolation and distinct environmental conditions can promote peripheral populations to diverge both genetically and phenotypically from core populations as a result of genetic drift and natural selection [1]. Peripheral populations are often smaller than core populations which can predestine such populations to bottleneck events [2]. This leads to the prediction that neutral genetic variations will be depleted in these populations. Nonetheless, peripheral populations may significantly contribute to a species' evolutionary potential by providing a source of adaptive genetic variance in a particular environmental setting [3, 4].

Taxa with island-like distribution patterns, such as the endemic cichlid fishes of the East African Great Lakes, provide an excellent opportunity for the study of evolutionary processes. Although many studies on these species have focussed on mechanisms of intralacustrine diversification within each of the main lakes [5, 6], the occurrence of endemic cichlids in Lake Nabugabo, a satellite lake isolated from Lake Victoria, initially raised the awareness of the evolutionary potential of peripheral isolates in the Great Lake region [7]. However, it was only recently that evidence was provided for the evolution of a cichlid species in an isolated satellite lake of Lake Malawi [8]. A genetically and phenotypically unique population of *Rhamphochromis*, a genus previously thought to be restricted to Lake Malawi and its permanently connected waters, was detected in Lake Chilingali. These examples suggest a role for (periodic) peripheral isolates in the evolutionary diversification of East African cichlids. In the present study, we evaluated empirical data on two economically important cichlid species to test whether peripheral populations from Lake Malombe, a satellite lake of Lake Malawi, have developed different genetic and phenotypic properties after recent colonisation from Lake Malawi.

Lake Malombe is a shallow, turbid, and nutrient-rich lake of about 390 km^2 with a maximum depth of only 5–7 m. The lake was formed by the recent (1930s) inundation of a flood plain of the Upper Shire River, about 15 km from its source in Lake Malawi [9]. Its ichthyofauna dominated by haplochromine cichlids is similar to that of Lake Malawi, but its diversity is lower and its relative productivity is higher. The lake supports an intensive fishery providing nearly 10% (ca. 4,000 t.y^{-1}) of the total yield (ca. 50–60,000 t.y^{-1}) within the Lake Malawi catchment [10, 11]. Since the mid-1980s, fishing effort on Lake Malombe has increased rapidly and mesh sizes have progressively been reduced to 19 mm or even less. Lake Malombe's haplochromine fishery yielded 9,500 tons in 1990 but this figure has declined to less than 4,000 tons in 2001 [12]. Among the economically most important haplochromines, *Copadichromis* sp. "*Virginalis kajose*" (cited as *C. virginalis*) landings declined by more than 50%, from 937 t.y^{-1} in 1991 to 412 t.y^{-1}in 2001, while in contrast, *C. chrysonotus* catches increased from about 120 t.y^{-1} to 510 t.y^{-1} over the same time period with a similar fishing effort [12].

Their semi-isolated state, the distinct habitat conditions and the elevated fishing pressure, may have caused the Lake Malombe populations of *Copadichromis* sp. "*Virginalis kajose*" and *C. chrysonotus* to be susceptible to genetic and phenotypic divergence from their conspecifics in the main lake. It has been shown that the genetic variability of a population can be substantially reduced as a result of a founder effect following colonization and/or elevated fishing mortality [13, 14]. To test for a depletion of the genetic variability in the Lake Malombe populations, we analysed mitochondrial DNA (mtDNA) sequence data and microsatellite DNA variation. MtDNA variation is particularly sensitive to bottlenecks and random fluctuations in genotype frequencies, as its effective population size is one-fourth of nuclear alleles [15]. Microsatellites may be particularly informative in detecting structure among recently colonized or geographically proximal populations because of the much higher mutation rate than for mtDNA [16]. Furthermore, we looked for morphometric differences between populations from lakes Malombe and Malawi in response to the distinct habitat conditions. Haplochromine cichlids have been shown to be morphologically exceedingly versatile in response to environmental factors [17–19]. We expected morphometric differences between populations from Lake Malombe and Lake Malawi to be higher than among populations within Lake Malawi.

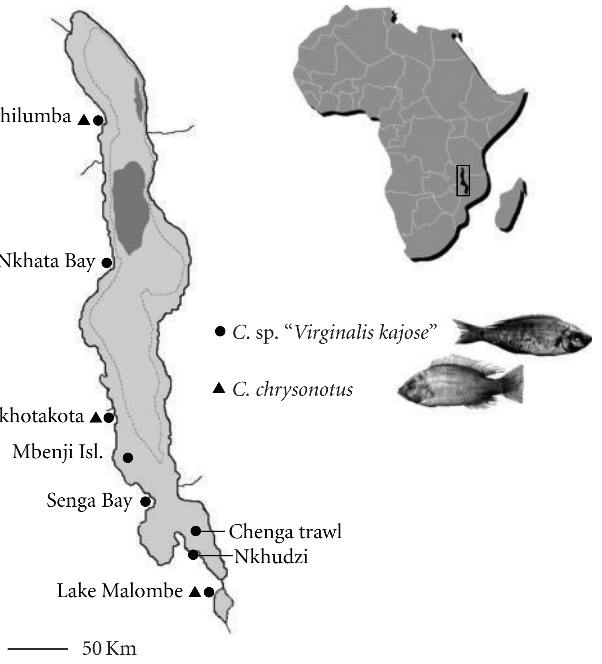


Figure 1: Map of Lakes Malawi and Malombe, East Africa. Sampling localities for *Copadichromis* sp. "*Virginalis kajose*" and *C. chrysonotus* are shown. Dashed bathymetric line indicates zones deeper than 250 metres; dark grey areas indicate lake zones deeper than 500 metres.

2. Materials and Methods

2.1. Sample Collection. A total of 427 *C.* sp. "*Virginalis kajose*" and 192 *C. chrysonotus* specimens were sampled from one site in Lake Malombe and, respectively, seven and two localities in Lake Malawi (Figure 1). All fishes were collected with open water seines (chirimila nets), purse seines (nkacha nets), and beach seines by artisanal fishermen, except for one sample of *C.* sp. "*Virginalis kajose*" that was caught by a commercial trawler. Pelvic fin clips were preserved in absolute ethanol and stored at room temperature. The specimens were fixed in 10% formalin and are curated at the Royal Museum for Central Africa in Tervuren, Belgium. All available samples were used in the analysis of microsatellite DNA variability, except for *C. chrysonotus* from Senga Bay because of its low sample size. A subset of ten to twelve samples per species per locality were sequenced for an analysis of mtDNA haplotype variation, except for *C.* sp. "*Virginalis kajose*" from Lake Malombe of which a total of 24 specimens were sequenced. For the morphological analysis, we examined approximately 20 specimens per species from each locality.

2.2. DNA Preparation and Amplification. Total genomic DNA was extracted using proteinase K digestion and salt precipitation, according to Aljanabi and Martinez [20]. DNA extracts were resuspended in 100 μL of autoclaved ddH$_2$O. All samples were examined for genetic variation at 6 microsatellite markers: Pzeb1, Pzeb3, Pzeb4, Pzeb5 [21], UNH002 [22], and TmoM11 [23]. PCRs were performed under the following conditions: 94°C for 120 s, followed by 5 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s, and followed by 30 cycles of 90°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. 10 μL reaction cocktails included 1 μL template DNA, 0.5 μM of each primer, 200 μM of each dNTP, 0.26 units Taq polymerase (Sigma Aldrich, Germany), and 1 μL 10x reaction buffer (Sigma Aldrich). PCR amplification products were run on 6% denaturing polyacrylamide gels using an ALF Express DNA Sequencer (Amersham Pharmacia Biotech). Fragment sizes were scored with ALFWin Fragment Analyser v1.0 (Amersham Pharmacia Biotech), using M13mp8 DNA standards as external references, following van Oppen et al. [21].

Mitochondrial DNA sequence variation was examined in a 325 bp fragment of the control region (D-loop). PCR amplification was carried out in a 25 μL buffered reaction mixture, containing 5 μL template DNA, 0.5 μM of each primer, H16498 and L15995 [24], 200 μM of each dNTP, 2.5 μL of 10x buffer (1 mM $MgCl_2$), and 0.65 units of Red Taq Polymerase (Sigma Aldrich). PCR conditions consisted of an initial denaturation at 94°C for 120 s, followed by 35 cycles of 94°C for 60 s, 52°C for 60 s, and 72°C for 120 s, followed by 72°C for 10 min. The success of amplification was checked by electrophoresis on 1% agarose gel. The PCR products were purified following the qiaquick PCR purification kit protocol, prior to being added as template for chain-termination sequencing, carried out in 10 μL reaction volumes, using 2 μL purified DNA template, 1 μL 5x Sequencing Buffer (Applied Biosystems), 2 μL Ready Reaction Mix (Applied Biosystems), and 2 μL primer (L15995, 2 μM). The sequencing program consisted of an initial denaturation step at 96°C for 60 s, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 240 s. DNA fragments were purified by ethanol precipitation and subsequently visualized on a 3130 capillary sequencer (Applied Biosystems).

2.3. Analysis of DNA Variation. Nuclear genetic diversity estimates, such as allele numbers and observed and unbiased expected number of heterozygotes at each locus, were calculated using GENETIX 4.02 [25]. Departures from Hardy-Weinberg equilibrium and linkage disequilibrium were tested using exact tests as implemented by GENEPOP 3.3 [26]. Significance levels were obtained by the Markov chain method using 5,000 dememorisations, 500 batches, and 2,000 iterations per batch. Allelic richness (AR) and estimates of global population differentiation using Wright's F_{ST} [27] and pairwise F_{ST} values, estimated by θ [28], were calculated in FSTAT 2.9.3 [29]. Significance of F_{ST} values was calculated in Genepop by the Markov chain method using 5,000 dememorisations, 2,000 permutations, and 500 batches in an exact test for population differentiation. Recent population size reduction was examined by testing for heterozygosity excess and mode shift in allele frequency with the program BOTTLENECK 1.2.02 [30]. Following the recommendations by Luikart and Cornuet [31] for microsatellite data, we used the Wilcoxon signed-rank test with a two-phased model of mutation (TPM). The parameters chosen for TPM were: variance 30% and probability for SMM 70%, and estimations were based on 1000 iterations.

After manual correction in Chromas 1.45, the mtDNA sequences were aligned using ClustalW [32] and visually checked afterwards. Haplotype frequencies, number of segregating sites, mean number of pairwise differences, gene diversity (H), and nucleotide diversity (π) were calculated with DNAsp [33]. For the two fish species, a mtDNA haplotype network was constructed with the program TCS (v. 1.21) using statistical parsimony [34].

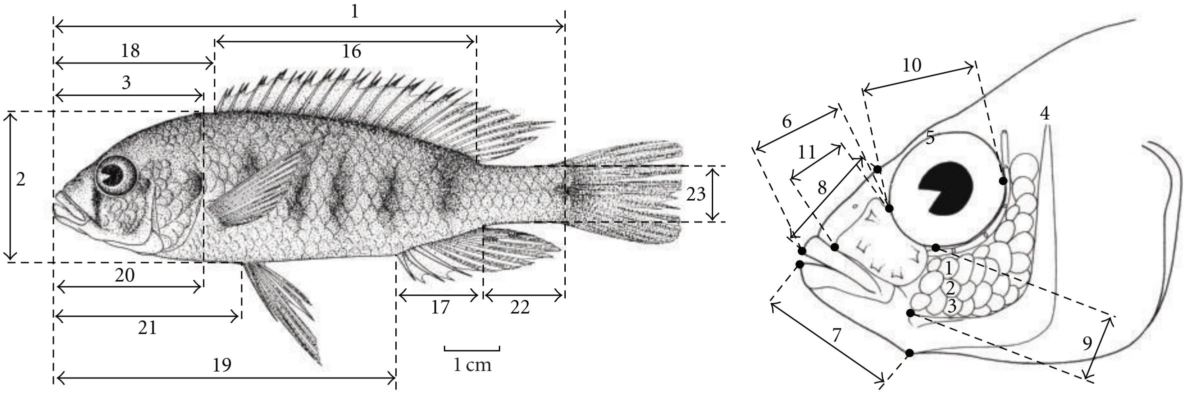
2.4. Analysis of Morphometric Variation. We measured 19 metric characters (Figure 2) as defined by Snoeks [35]. The measurements were taken with dial callipers under a binocular microscope. The 19 measurements were standard length (SL), body depth (BD), head length (HL), head width (HW), interorbital width (IOW), snout length (SnL), lower jaw length (LJL), premaxillary pedicel length (PPL), cheek depth (ChD), eye diameter (ED), lacrimal depth (LaD), dorsal fin base length (DFB), anal fin base length (AFB), predorsal fin distance (PRD), preanal fin distance (PRA), prepectoral fin distance (PRP), preventral fin distance (PRV), caudal peduncle length (CPL) and caudal peduncle depth (CPD). These measurements are typically used in taxonomic research on cichlids, where they have proven to be useful to detect small phenotypic differences, even among conspecific populations [35].

Data were explored and analysed using principal component analysis on the log-transformed measurements. A covariance matrix was used to calculate the loadings of the variables and the scores of the specimens on the principal component axes. Loadings of the variables on the first principal component (PC1) were of the same sign and of a similar magnitude, indicating that this axis can be interpreted as a proxy for general size [36]. All computations were performed in the R software v. 2.11.1 [37].

3. Results

3.1. Genetic Differences. In *C.* sp. "*Virginalis kajose,*" allelic richness (*AR*) ranged from 16.46 (Chilumba) to 19.00 (Chenga Trawler), mean number of alleles per locus (*Na*) from 17.7 (Chilumba) to 21.7 (Mbenji Island), observed heterozygosity (H_O) from 0.5929 (Nkhudzi Bay) to 0.7326 (Nkhotakota), and average expected heterozygosity (H_E) from 0.7541 (Nkhata Bay) to 0.7854 (Senga Bay) for the Lake Malawi populations (Table 1). The population from Lake Malombe showed intermediate AR (16.92), *Na* (18.2), and H_O (0.6195) and a lower H_E (0.7375) compared to the Lake Malawi populations. In *C. chrysonotus*, all measures of genetic diversity (*Na*, AR, H_E) except for H_O were consistently lower in the Lake Malombe population compared to the Lake Malawi populations (Table 1).

In *C.* sp. "*Virginalis kajose,*" 18 of 48 single-locus tests and in *C. chrysonotus* 7 of 18 single-locus tests for deviation from Hardy-Weinberg equilibrium were significant at a table-wide Bonferroni-corrected alpha level ($\alpha_{corr} = 0.001$ and $\alpha_{corr} = 0.002$ for the two species, resp.) (Table 1). Most deviations occurred across populations at loci Pzeb1, UNH002, and TmoM11, all involving heterozygote deficits. Loci Pzeb3, Pzeb4, and Pzeb5 were in agreement with Hardy-Weinberg equilibrium expectations in 6 of 33 single-locus tests (Table 1). Linkage disequilibrium tests across loci and



(a) (b)

FIGURE 2: Overview of the morphometric measurements used in our study. Enumeration following Snoeks (2004). 1 = standard length (SL); 2 = body depth (BD); 3 = head length (HL); 4 = head width (HW); 5 = inter orbital width (IOW); 6 = snout length (SnL); 7 = lower jaw length (LJL); 8 = premaxillary pedicel length (PPL); 9 = cheek depth (ChD); 10 = eye diameter (ED); 11 = lacrimal depth (LaD); 16 = dorsal fin base length (DFB); 17 = anal fin base length (AFB); 18 = predorsal fin distance (PRD); 19 = preanal fin distance (PRA); 20 = prepectoral fin distance (PRP); 21 = preventral fin distance (PRV); 22 = caudal peduncle length (CPL); 23 = caudal peduncle depth (CPD).

TABLE 1: Genetic diversity in two *Copadichromis* species, genotyped at six unlinked microsatellite loci. *N*: sample size; *Na*: mean number of alleles per locus (direct count); *AR*: allelic richness (i.e., standardized for minimal sample size 40 and 53); *Ho*: observed heterozygosity; *He*: unbiased expected heterozygosity. For each population, the *P* values for the single-locus tests for departure from Hardy-Weinberg equilibrium are tabulated. Populations: Chilumba (CH), Nkhata (NK), Nkhotakota (NO), Mbenji Island (MI), Senga Bay (SB), Chenga trawl (CT), Nkhudzi Bay (NKU), and Lake Malombe (LM).

(a) *Copadichromis* sp. "*Virginalis kajose*"

	Lake Malombe	Lake Malawi						
		CT	NKU	SB	MI	NO	NK	CH
N	**53**	40	61	63	57	48	54	51
Na	**18.2**	19.0	19.8	20.5	21.7	20.0	18.7	17.7
AR	**16.92**	19.00	17.59	18.17	18.87	18.88	16.89	16.46
Ho	**0.6195**	0.7083	0.5929	0.7090	0.6871	0.7326	0.6821	0.683
He	**0.7375**	0.777	0.7729	0.7854	0.7648	0.7614	0.7541	0.7493
Pzeb1	<**0.0001**	0.0792	<0.0001	0.0143	0.0179	<0.0001	0.0021	<0.0001
Pzeb3	**0.1075**	0.2054	<0.0001	0.0052	0.1802	0.8029	0.0306	0.3968
Pzeb4	**0.0160**	0.4785	0.0141	0.0161	<0.0001	0.2977	0.4464	0.0625
Pzeb5	**0.0001**	0.0030	<0.0001	0.6380	0.6631	0.1240	1.0000	0.3285
UNH002	<**0.0001**	<0.0001	0.0068	0.4499	<0.0001	1.0000	0.0295	0.0413
TmoM11	<**0.0001**	0.5857	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

(b) *Copadichromis chrysonotus*

	Lake Malombe	Lake Malawi	
		NO	CH
N	**54**	65	53
Na	**13.7**	18.5	15.8
AR	**13.61**	17.51	15.80
Ho	**0.6574**	0.6718	0.6132
He	**0.7265**	0.7681	0.7776
Pzeb1	<**0.0001**	0.0029	0.0012
Pzeb3	**0.1486**	0.1504	<0.0001
Pzeb4	**0.6093**	0.1347	0.0305
Pzeb5	**0.0104**	<0.0001	<0.0001
UNH002	**0.0146**	0.0503	0.0002
TmoM11	**0.5840**	0.1782	<0.0001

Table 2: Pairwise values of F_{ST} (below the diagonal) and associated P values (above the diagonal) among eight *C.* sp. "*Virginalis kajose*" populations (codes following Table 1).

	Lake Malombe	Lake Malawi						
		CT	NKU	SB	MI	NO	NK	CH
LM	—	<0.0001	0.0021	<0.0001	<0.0001	0.2393	0.0002	<0.0001
CT	0.009	—	<0.0001	0.0253	0.0807	0.0002	0.0451	<0.0001
NKU	0.005	0.007	—	<0.0001	<0.0001	0.0006	0.0178	<0.0001
SB	0.007	0.001	0.007	—	<0.0001	0.0055	0.0688	<0.0001
MI	0.010	0.002	0.006	0.005	—	<0.0001	0.0424	<0.0001
NO	0.003	0.011	0.011	0.007	0.011	—	<0.0001	<0.0001
NK	0.006	0.007	0.004	0.001	0.002	0.008	—	<0.0001
CH	0.011	0.010	0.015	0.010	0.008	0.013	0.009	—

Table 3: Pairwise values of F_{ST} (below the diagonal) and associated P values (above the diagonal) among three *C. chrysonotus* populations (codes following Table 1).

	Lake Malombe	Lake Malawi	
		NO	CH
LM	—	<0.0001	<0.0001
NO	0.01547	—	<0.0001
CH	0.01430	0.00780	—

populations revealed no significant allelic associations in *C.* sp. "*Virginalis kajose*" nor in *C. chrysonotus* (all $P > .05$).

Microsatellite data provided no evidence for a recent bottleneck in both species. We found no significant excess in gene diversity in any population. Concordantly, the mode-shift tests implemented in *bottleneck* showed no shift in the frequency distributions of microsatellite alleles. As expected for nonbottlenecked populations, all populations showed L-shaped allele frequencies.

In both species, we detected low but significant population structuring. The global measure for population differentiation across Lake Malombe populations was $F_{\mathrm{ST}} = 0.005$ ($P < .001$) in *C.* sp. "*Virginalis kajose*" and $F_{\mathrm{ST}} = 0.008$ ($P < .001$) in *C. chrysonotus.* Pairwise F_{ST} values are presented in Tables 2 and 3. For *C.* sp. "*Virginalis kajose,*" the measures for genetic differentiation between the Lake Malombe population and the populations from Lake Malawi were of the same order of magnitude as the genetic differentiation among populations within Lake Malawi (Table 2). For *C. chrysonotus*, the level of genetic differentiation between the Lake Malombe population and the Lake Malawi populations was higher than the level of genetic differentiation among the Lake Malawi populations (Table 3).

In the complete mtDNA dataset of 135 sequences, a total of 42 variable sites ($n_{\mathrm{total}} = 325\,\mathrm{bp}$) resulted in 40 haplotypes. The 98 sequences of *C.* sp. "*Virginalis kajose*" contained 36 polymorphic sites resulting in 32 haplotypes (Figure 3). The 37 *C. chrysonotus* sequences with 18 variable sites represented 9 haplotypes (Figure 4). Gene diversity in *C.* sp. "*Virginalis kajose*" ranged from 0.47 (Senga Bay) to 0.87 (Nkhudzi Bay) (Table 4). In *C. chrysonotus*, gene diversity was markedly lower ranging from zero (Lake Malombe) to 0.67 (Nkhotakota). Nucleotide diversity varied from 0.0037 (Nkhotakota) to 0.0263 (Nkhudzi Bay) in *C.* sp. "*Virginalis kajose*" and from zero (Lake Malombe) to 0.0180 (Nkhotakota) in *C. chrysonotus*. The only haplotype shared between the two species occurred in a single *C.* sp. "*Virginalis kajose*" from Lake Malombe (indicated with an asterisk in Figure 3) and in 28 *C. chrysonotus* from Lake Malombe ($n = 13$), Nkhotakota ($n = 7$,) and Chilumba ($n = 8$, indicated with an asterisk in Figure 4). Of the seven haplotypes found in *C.* sp. "*Virginalis kajose*" from Lake Malombe, six are also present in Lake Malawi, which contained 30 haplotypes in total. The haplotype that is unique to Lake Malombe differs by a single mutation (T to C) from a widespread haplotype, present in five populations from Lake Malawi.

3.2. Body Size Differences. In both species, the specimens from Lake Malombe were smaller with an average SL of 55.1 ± 2.3 mm and 73.1 ± 6.8 mm for *C.* sp. "*Virginalis kajose*" and *C. chrysonotus*, respectively, compared to 90.2 ± 12.4 mm and 108.7 ± 11.4 mm in the populations from Lake Malawi.

3.3. Morphometric Differences. For *C.* sp. "*Virginalis kajose,*" PCA on the log-transformed measurements did not allow us to discriminate the Lake Malombe population from the Lake Malawi populations. The individual principal component scores overlapped largely on all PC axes for the specimens from both lakes (MANOVA, $F_{(17,113)} = 1.0889$, $P = .3731$), except on PC1 which is a proxy for size. This overlap is illustrated for the scores on PC2 and PC3 in Figure 5. The variables that loaded most on PC2 were anal fin base length, caudal peduncle depth, body depth and eye diameter (Supplementary Table 1, see in Supplementary Material available at doi: 10.4061/2011/835946).

For *C. chrysonotus,* the specimens from Lake Malombe could not be distinguished from the Lake Malawi specimens by their scores on the principal component axes (MANOVA, $F_{(17,53)} = 0.4062$, $P = .9781$; see Figure 6). The variables that loaded most with PC2 were eye diameter, anal fin base

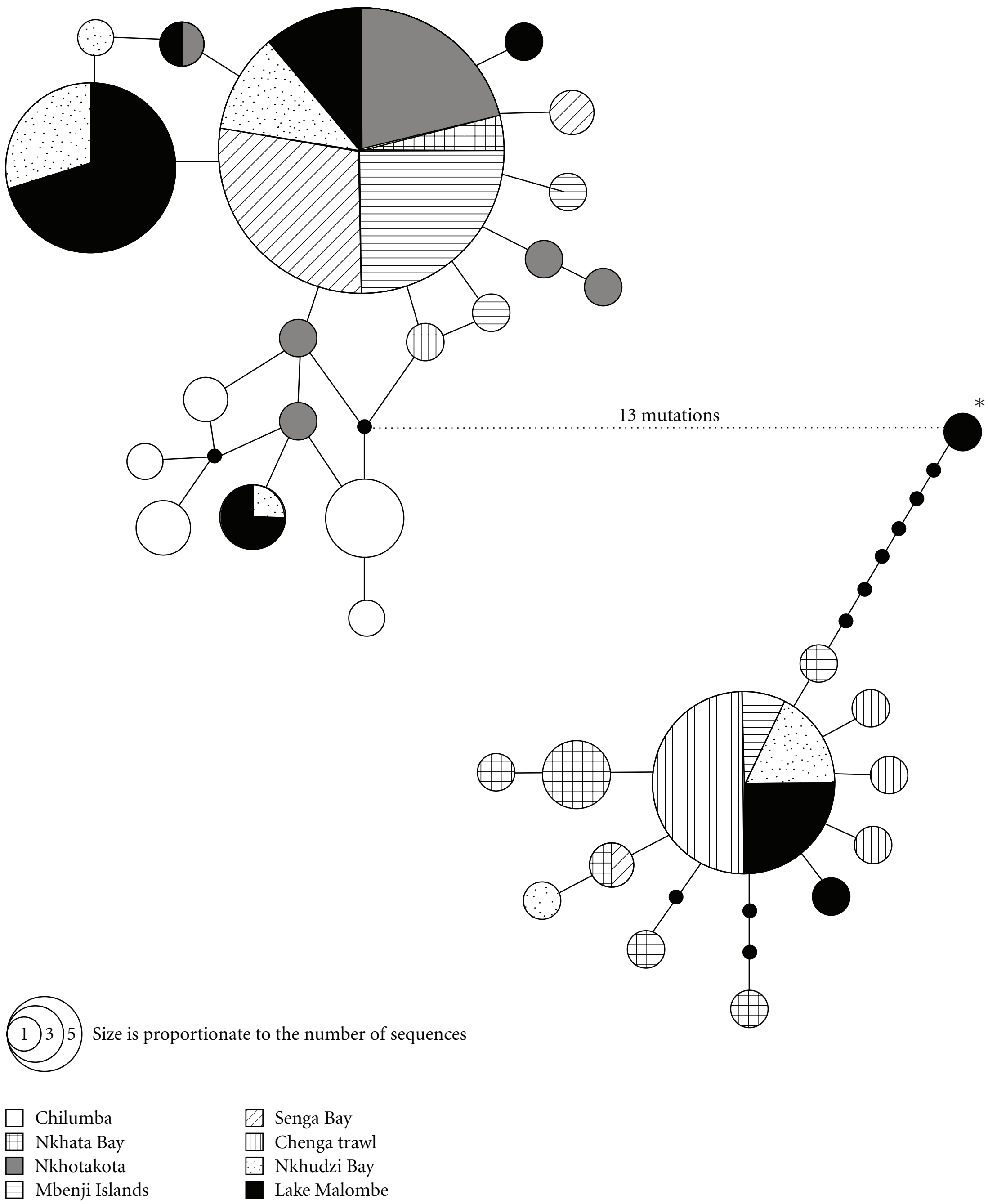


Figure 3: Haplotype network for the *C.* sp. "*Virginalis kajose*" sequences (based on 325 bp fragments of the mtDNA control region). The haplotype indicated with an asterisk is the haplotype that is shared with *C. chrysonotus* (see Figure 4).

length, lower jaw length, body depth, and caudal peduncle depth (Supplementary Table 2).

4. Discussion

4.1. Genetic Differentiation. Peripheral populations at the edge of a species' range are often less numerous than and isolated from core populations. Consequently, gene flow will be reduced, and stochastic demographic events such as bottlenecks, founder effects, and genetic drift may lead to a substantial loss of multilocus heterozygosity and allelic variation [1–3, 38]. In addition, elevated fishing mortality may also lead to a reduction in genetic variability perhaps owing to a selection response [39, 40], though at present the evidence of genetic change resulting from fishing is not unequivocal [41]. Populations of *Copadichromis* sp. *Virginalis kajose* and *C. chrysonotus* from Lake Malombe were expected to present reduced genetic variability when compared to their source populations in Lake Malawi because of their semi-isolated state, the distinct habitat

Table 4: Genetic diversity in two *Copadichromis* species based on a 325 bp mtDNA D-loop sequence. Sampling sites encoded as in Table 1. Genbank accession numbers for *Copadichromis* sp. "*Virginalis kajose*": EF211832-211842, EF211848-211859, EF211868-211878, and EF647209-647272; Genbank accession numbers for *C. chrysonotus*: EF647273-647309, and EF647320-647331.

(a) *Copadichromis* sp. "*Virginalis kajose*"

	Lake Malombe	Lake Malawi						
Population code	LM	CT	CH	NO	NK	MI	SB	NKU
Sample size	21	10	12	11	10	12	11	11
Number haplotypes	7	5	5	6	7	6	3	6
Number polymorphic sites	22	17	6	5	22	15	15	19
Gene diversity	0.810 ± 0.004	0.667 ± 0.027	0.788 ± 0.008	0.727 ± 0.021	0.867 ± 0.011	0.682 ± 0.022	0.473 ± 0.026	0.873 ± 0.005
Average pairwise difference	6.981	3.4000	2.136	1.200	4.867	3.045	2.873	7.673
Nucleotide diversity (average over loci)	0.02161 ± 0.00002	0.01053 ± 0.00004	0.00659 ± 0.00001	0.00370 ± 0.00001	0.01507 ± 0.00005	0.00943 ± 0.00003	0.00889 ± 0.00004	0.02375 ± 0.00003

(b) *Copadichromis chrysonotus*

	Lake Malombe	Lake Malawi	
Population code	LM	CH	NO
Sample size	13	12	12
Number haplotypes	1	5	5
Number polymorphic sites	0	15	15
Gene diversity	0	0.576 ± 0.027	0.667 ± 0.020
Average pairwise difference	0	3.727	5.833
Nucleotide diversity (average over loci)	0	0.01150 ± 0.00003	0.0180 ± 0.00002

conditions and elevated fishing pressure in the peripheral Lake Malombe.

We found low levels of population substructuring both within and among lakes, indicating that the studied fish species are relatively mobile and suggesting recent or ongoing gene flow between Lake Malawi and Lake Malombe populations. Lake Malombe is believed to have refilled around the 1930s [9], so the Lake Malombe population represents a relatively recent peripheral isolate. Furthermore, it has been shown that the Upper Shire River is used by various fish species migrating between the lakes [42], and this could probably also be the case for the two studied *Copadichromis* species. Despite the low degree of population subdivision, both species yielded a slightly decreased genetic diversity in Lake Malombe, especially for *C. chrysonotus*. We assume that the observed diminution in genetic variability is not caused by the high fishing mortality, because if true, we would rather expect a significant loss of genetic variation in *C.* sp. "*Virginalis kajose*" for which catch sizes in Lake Malombe have already declined [12]. Instead, a founder effect, that is, a population bottleneck associated with the colonization of Lake Malombe, may have been of principal importance for the observed lower genetic variation in these populations. Our microsatellite data did not however indicate for a heterozygosity excess nor a mode shift in the allele distribution pattern. Population bottlenecks of short duration are expected to substantially reduce the number of alleles present while having less effect on multilocus heterozygosity [43]. Moreover, our low sample sizes with respect to the high allelic richness may have lead to an artificial large proportion of low-frequency allele classes resulting in L-shaped allele frequency distributions and could therefore have influenced the robustness of the mode-shift test. Also, it is important to note that 18 of 48 single-locus tests in *C.* sp. "*Virginalis kajose*" and 7 of 18 in *C. chrysonotus* showed a deficit in heterozygotes. Most of the observed Hardy-Weinberg disequilibria were detected at three loci (Pzeb1, UNH002, and TmoM11) and are likely the result of the presence of nonamplifying null alleles (see [44] and references therein).

Inspection of the distribution pattern of mtDNA haplotypes provides support for the hypothesis of a natural founder effect with a different impact on each species. The single mtDNA haplotype detected in *C. chrysonotus* from Lake Malombe is the most common haplotype found for that species in Lake Malawi, with frequencies that exceed

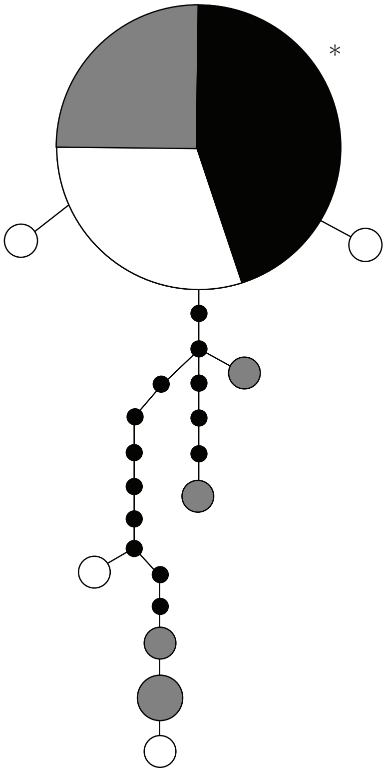


Figure 4: Haplotype network for the *C. chrysonotus* sequences (based on 325 bp fragments of the mtDNA control region). The haplotype indicated with an asterisk is the haplotype that is shared with *C.* sp. "*Virginalis kajose*" (see Figure 3). The legend is given in Figure 3.

58% in each of the sampled populations. Consequently, a stochastic shift in haplotype variation associated with the founding event, or genetic drift following colonization, may have resulted in the presence of only this haplotype in Lake Malombe for *C. chrysonotus*. Following the same reasoning, it appears logic that the change in haplotype variation was less pronounced for *C.* sp. "*Virginalis kajose*" because of the generally higher haplotype diversity in its source populations from Lake Malawi ($H_{\text{mean}} = 0.725$ versus $H_{\text{mean}} = 0.520$ for *C. chrysonotus*). Moreover, of the six haplotypes detected in the southernmost *C.* sp. "*Virginalis kajose*" population (Nkhudzi Bay), only the four most abundant ones are found in Lake Malombe.

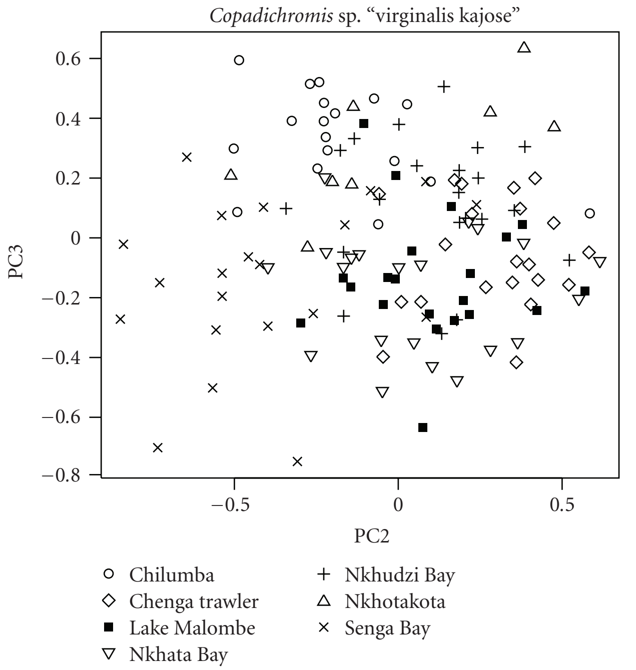


Figure 5: Scatterplot of scores on the second and third axes of a PCA on log-transformed measurements for *Copadichromis* sp. "*Virginalis kajose irginalis kajose*". The scores of the Lake Malombe individuals fall within the range of the scores for the Lake Malawi individuals.

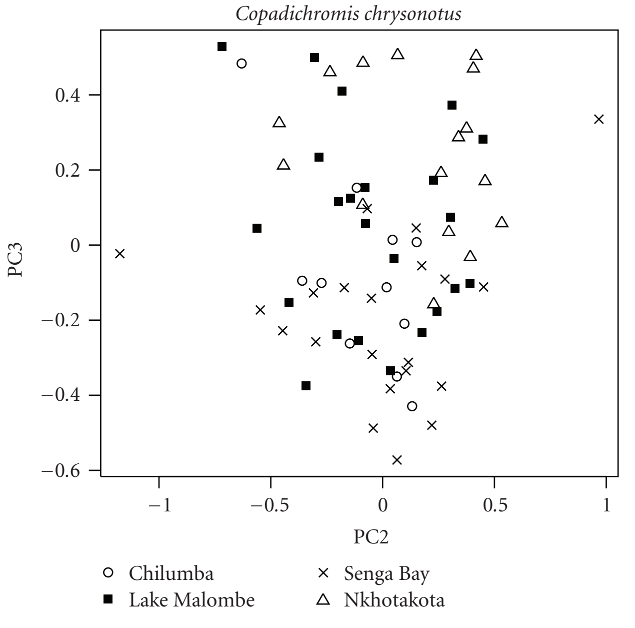


Figure 6: Scatterplot of scores on the second and third axes of a PCA on log-transformed measurements for *Copadichromis chrysonotus*. The scores of the Lake Malombe individuals fall within the range of the scores for the Lake Malawi individuals.

4.2. Body Size Differences. Reduction in size at maturity is a predictive outcome of fishing pressure, either through fisheries-induced evolution or through phenotypic plasticity [45–47]. Reduction in body size was observed for heavily fished cichlid species, such as tilapia [48] and haplochromines from Lake Victoria [49, 50]. For both species in this study, the mean body size was significantly smaller in Lake Malombe than in Lake Malawi, which is in agreement with the hypothesis of a fishery pressure effect, although we cannot exclude the possibility that body size is affected by environmental effects. In any case, it is unlikely that the differences in body sizes are due to possible sampling artefacts, as our results are consistent with body sizes reported by Weyl et al. [12] from repetitive samples from Lake Malombe between March 2000 and April 2001.

4.3. Morphological Differentiation. Mayr [3] pointed out that populations at the edge of a species' range often diverge in morphology from central populations. Peripheral populations are likely to experience different regimes of ecological selection than central populations, often leading to adaptive divergence [4, 51]. In East African haplochromine cichlids, ecological adaptation is suggested to be one of the major mechanisms behind their explosive radiation [5, 52–54]. As such, smaller isolated peripheral water bodies may be of significant importance to the diversification of cichlid species in the East African Great Lakes by generating novel phenotypes in allopatry [3, 4, 8].

Lake Malombe's distinct habitat conditions (e.g., its shallow, eutrophic, turbid water state in contrast to the great visibility in the much deeper Lake Malawi [9]) would suggest an adequate basis for ecological differentiation leading to the generation of local phenotypes. Nonetheless, we found that peripheral populations of two *Copadichromis* species in Lake Malombe could not be distinguished morphologically from central populations in Lake Malawi. Theory predicts that adaptive divergence between populations reflects a balance between the diversifying effects of local selection and the homogenizing effects of gene flow [4, 55, 56]. Although morphological differentiation can occur in the face of gene flow, particularly when divergent selection is strong (see [57] and references therein), gene flow will often constrain morphological divergence [56]. The low degree of population subdivision as revealed by our microsatellite data seems to support the idea that gene flow between Lake Malombe and Lake Malawi populations is too substantial to allow for morphological divergence. Alternatively, it could also be that ecological divergence here is not manifested by divergence in shape, but rather by (e.g., physiological) adaptations which could not be perceived by our measurements.

5. Conclusion

Young peripheral populations of two *Copadichromis* species in Lake Malombe, connected to Lake Malawi via the Shire River, showed low genetic divergence and were morphometrically not distinct from their source populations in the main lake. This may be attributed to either homogenizing ongoing gene flow through the Shire River or very recently time since isolation of the populations in the satellite lake. Nonetheless, evidence was found for a founder event with a different impact on the genetic composition of the two species. We did not find direct evidence for a fisheries-induced impact on the genetic variability. Instead, body size for both species was significantly reduced in Lake Malombe, which could be a consequence of high fishing pressure and the use of small mesh sizes.

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Research Article

Community Genetics Reveal Elevated Levels of Sympatric Gene Flow among Morphologically Similar but Not among Morphologically Dissimilar Species of Lake Victoria Cichlid Fish

N. Konijnendijk,[1, 2, 3] D. A. Joyce,[2, 4] H. D. J. Mrosso,[2, 5] M. Egas,[3] and O. Seehausen[1, 2]

[1] *Institute of Ecology and Evolution, University of Bern, Baltzerstrasse 6, CH-3012 Bern, Switzerland*
[2] *EAWAG, Center for Ecology, Evolution and Biogeochemistry (CEEB), Seestrasse 79, CH-6047 Kastanienbaum, Switzerland*
[3] *Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands*
[4] *Department of Biological Sciences, University of Hull, Hull HU6 7RX, UK*
[5] *Tanzanian Fisheries Research Institute, P.O. Box 475, Mwanza, Tanzania*

Correspondence should be addressed to O. Seehausen, ole.seehausen@eawag.ch

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We examined genetic structure among five species of Lake Victoria haplochromine cichlids in four island communities, using a full factorial sampling design that compared genetic differentiation between pairs of species and populations of varying morphological similarity and geographical proximity. We found that allopatric conspecific populations were on average significantly more strongly differentiated than sympatric heterospecific populations of morphologically similar species. Allopatric heterospecific populations of morphologically dissimilar species were most differentiated. Our work demonstrates that phenotypic divergence can be maintained and perhaps even evolve in sympatry despite considerable gene flow between species. Conversely, phenotypic resemblance among conspecific populations can be maintained despite geographical isolation. Additionally we show that anthropogenically increased hybridization does not affect all sympatric species evenly but predominantly affects morphologically similar and closely related species. This has important implications for the evolution of reproductive isolation between species These findings are also consistent with the hypothesis of speciation reversal due to weakening of divergent selection and reproductive isolation as a consequence of habitat homogenization and offers an evolutionary mechanistic explanation for the observation that species poor assemblages in turbid areas of the lake are characterized by just one or two species in each of a few morphologically distinct genera.

1. Introduction

How common gene flow is in nature among young, yet morphologically and ecologically distinct species, and how much phenotypic differentiation can be maintained in its face, is subject of considerable debate [1–3]. Investigating young adaptive radiations can be illuminating in this regard [4, 5]. In the history of life, adaptive radiations were an important source of species diversity, being bursts of speciation associated with ecological diversification often without major geographical barriers [6]. Hence, understanding the constraints to speciation and species coexistence in adaptive radiations may help understand the evolutionary structure of biodiversity more generally. The Lake Victoria cichlid species flock is one of the largest and fastest of all known adaptive radiations. With at least 500 species of cichlid fish, most of which most probably arose after the last desiccation of the lake, 15.000 years ago [7–9], this is an extraordinarily young radiation [10]. The more than 100 species of rock bottom-dwelling cichlids in Lake Victoria are defined by a combination of differences in coloration and morphological differences that often coincide with ecological differentiation.

Divergence among these species is so recent that intrinsic isolation seems almost completely absent [11, 12].

In the similar but older Lake Malawi mbuna radiation, Rico et al. [13] found little evidence of recent gene flow among sympatric species. Genetic distances among conspecific allopatric populations were generally smaller than among sympatric heterospecific populations, and the latter were no lower than those between allopatric heterospecific populations. This suggested speciation was not very recent and was followed by range expansion with little or no gene flow in secondary sympatry. The mbuna radiation is about 0.48 million years old [14, 15], and the lake has undergone many lake level fluctuations that would separate and reunite habitat patches, perhaps permitting time for allopatric origins of rather strong reproductive barriers among some species despite considerable evidence for historical [15] and recent [16, 17] gene flow among other species. Given the very short history of the Lake Victoria radiation, allopatric origins of strong reproductive barriers would seem less likely in Lake Victoria. Samonte et al. [18] used 11 genetic markers to investigate the genetic structure between four species of the Lake Victoria species flock. These species were considerably differentiated in ecology and morphology, but Samonte et al. [18] did not find any statistically significant signal of genetic structure amongst them, leading the authors to suggest that the Lake Victoria flock may consist of one large gene pool without real species.

We studied five sympatric putative species of Lake Victoria cichlids varying in their morphological similarity and probably in their relatedness, at four locations near rocky shore islands in the Mwanza region (Tanzania, Figure 1). The Mwanza Gulf is characterized by a strong North-South gradient in water turbidity (turbid in the South, relatively clear in the North). The existence of the gradient is probably natural, or at least was already present almost 100 years ago [19], but recent anthropogenic eutrophication has intensified and steepened the gradient [20]. Putatively closely related (i.e., congeneric, where genera are morphologically defined) sympatric species are more numerous [21], and have in one case been shown to be also genetically more distinct in clearer waters in the North, but previous genetic data were restricted to microsatellite DNA in one sister species complex, *Pundamilia pundamilia* and *P. nyererei* [20]. Here, we extend this to take a community genetics approach. We used a larger number of genomic amplified fragment length polymorphisms (AFLPs) to infer phylogenomic relationships, genetic differentiation and gene flow within and between island locations among five morphologically differentiated species that coexist at all of these islands and together make the numerically dominant component of each of these communities. AFLPs have proven powerful in resolving population genetic and phylogenetic structure in cichlid fish radiations [22–26]. Fixation indices were compared between groups of population pairs representing morphologically defined clades and geographical coincidence, allowing us to compare effects of spatial proximity on morphologically similar and dissimilar taxa.

2. Materials and Methods

2.1. Sampling. Individuals of five sympatric species of rock-dwelling haplochromine cichlids were collected by angling and gill netting around four little islands in the Mwanza Gulf between February and April 2005 (Figure 1). Only males were used for this study due to the difficulty of identifying females reliably to species level for some of the species. At the locations close to Luanso and Marumbi islands where intermediate phenotypes between the blue *Pundamilia pundamilia* and the red *P. nyererei* are common, we selected typical blue and typical red males from a large sample of males. Intermediate phenotypes between *N. greenwoodi* and *M. mbipi* were also common there, and we proceeded in the same way, using the characteristics described for these two species from other islands [27] to classify individuals as *N. greenwoodi*-like and *M. mbipi*-like. Of each fish a digital picture was taken immediately after capture in a photo cuvette and fin clips were taken, preserved in 95% ethanol and stored at −20°C. We collected, preserved and identified all cichlids species caught at our sampling locations during our sampling campaign in spring 2005. This allowed us to examine community composition by quantifying species abundance.

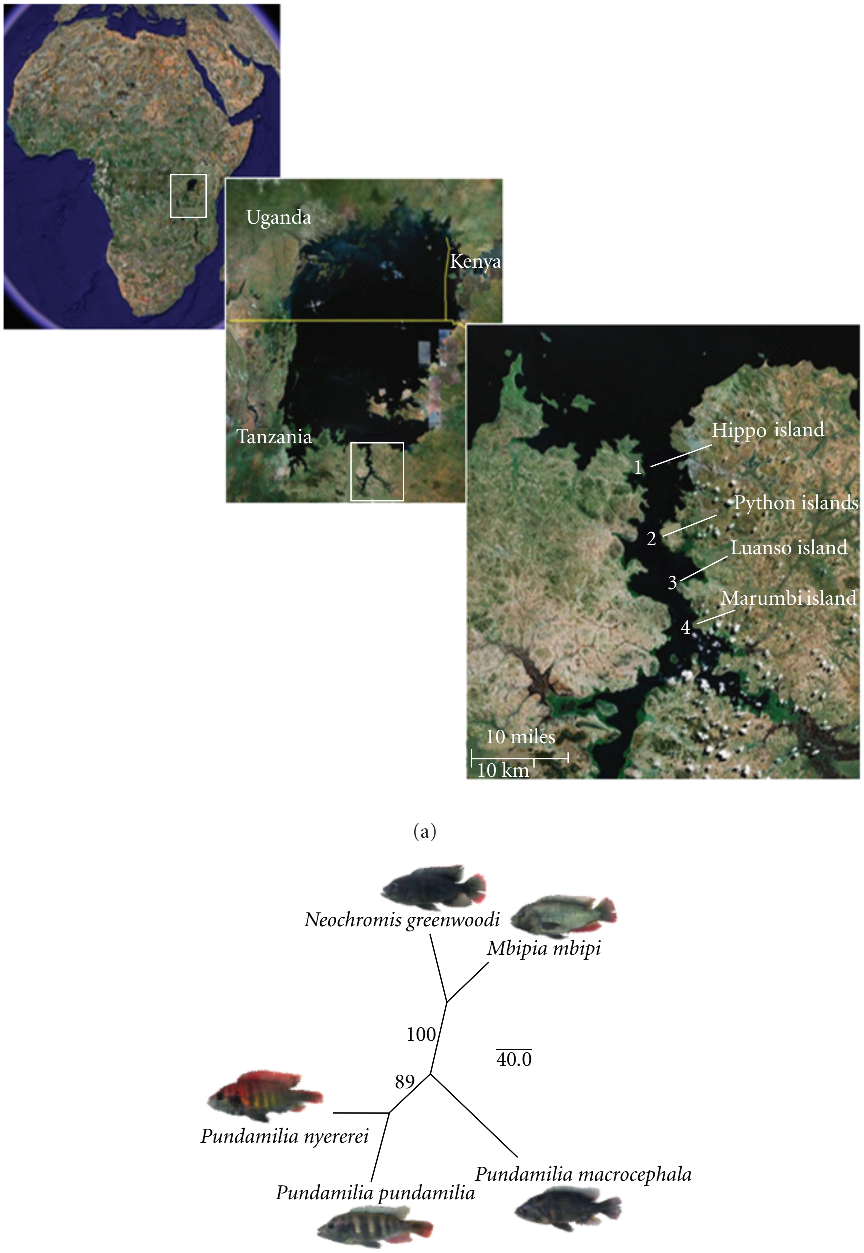
2.2. Choice of Taxa. The five species were selected because they were all present at each of the four sampling locations and represented two morphologically defined clades [27]: (1) the blue and red sister species *Pundamilia pundamilia* and *P. nyererei* and (2) the morphologically similar species pair *Neochromis greenwoodi* and *Mbipi mbipi*. Finally, *Pundamilia macrocephala* is morphologically more closely related to *P. pundamilia* and *P. nyererei* than to *M. mbipi* and *N. greenwoodi* but had never been studied genetically and closely resembles the latter two in its male nuptial coloration [27, 28]. These five species include the most abundant species in each of the four assemblages (Figure 2). The number of individuals that were genotyped for each of the five species ranged from four to eleven individuals per location (Table 1).

2.3. DNA Extraction and AFLP Analysis. DNA of all samples was purified with a standard phenol-chloroform procedure. Subsequently, the AFLP method by Vos et al. [29] was used for further steps, with minor modifications involving the use of four fluorescence-labeled primer combinations (*Mse*I-CAT/*Eco*RI-AAG (green), *Mse*I-CTA/*Eco*RI-AAG (green), *Mse*I-CAT/*Eco*RI-ACC (blue), and *Mse*I-CTA/*Eco*RI-ACC (blue)). Selective amplification products were separated on a Beckman Coulter CEQ 8000 capillary system.

2.4. Band Calling and Binning. Traces were analyzed using the automatic binning procedure and checking each fragment by eye in the Fragment Analysis program of the CEQ 8000 software. We scored fragments between 60 and 260 base pairs to ensure that there were no erroneous bands between samples due to differential and unreliable amplification of larger alleles. AFLP fragments were scored as dominant markers that could either be absent (0) or present (1). Slope

TABLE 1: F_{ST} estimates for all pairwise population comparisons (below diagonal) and corresponding P values (above diagonal). Significant F_{ST} estimates are also marked with 1 star ($P < 0.05$), 2 stars ($P < 0.01$), or 3 stars ($P < 0.001$). F_{ST} estimates were obtained with Arlequin 2.0. Cell colours indicate type of comparison: blue: allopatric conspecific; olive: sympatric sister species; green: allopatric sister species; yellow: sympatric nonsister species; white: allopatric nonsister species.

	M. mbipi Hippo	*M. mbipi* Python	*M. mbipi* Luanso	*M. mbipi* Marumbi	*N. greenwoodi* Hippo	*N. greenwoodi* Python	*N. greenwoodi* Luanso	*N. greenwoodi* Marumbi	*P. macrocephala* Hippo	*P. macrocephala* Python	*P. macrocephala* Luanso	*P. macrocephala* Marumbi	*P. nyererei* Hippo	*P nyererei* Python	*P. nyererei* Luanso	*P. nyererei* Marumbi	*P. pundamilia* Hippo	*P. pundamilia* Python	*P. pundamilia* Luanso	*P. pundamilia* Marumbi
M. mbipi Hippo (n=7)	—	0.00614	0.00733	0.01208	0.00545	0.00010	0.00010	0.09702	0.02366	0.00010	0.00683	0.00010	0.02336	0.00050	0.00000	0.00950	0.00069	0.00040	0.00000	0.00228
M. mbipi Python (n=8)	0.07133**	—	0.00010	0.00020	0.00000	0.09870	0.00000	0.00000	0.03584	0.00000	0.00574	0.00109	0.00277	0.00000	0.00000	0.00188	0.07910	0.00010	0.00000	0.00238
M. mbipi Luanso (n=11)	0.04268**	0.08904***	—	0.07415	0.00000	0.21097	0.43788	0.10454	0.09108	0.01554	0.01931	0.00792	0.00158	0.00297	0.00000	0.05475	0.00000	0.00020	0.00000	0.00099
M. mbipi Marumbi (n=9)	0.04075*	0.09115***	0.02001	—	0.00000	0.00040	0.00000	0.12781	0.09197	0.00000	0.00020	0.00020	0.00079	0.00000	0.00000	0.00000	0.00000	0.00010	0.00000	0.00168
N. greenwoodi Hippo (n=10)	0.03996**	0.12948***	0.06354***	0.07948***	—	0.00000	0.00000	0.00010	0.11207	0.00010	0.01129	0.00040	0.00050	0.00020	0.00000	0.00119	0.00059	0.00000	0.00020	0.00069
N. greenwoodi Python (n=10)	0.07620***	0.04195	0.01218	0.06232***	0.06510***	—	0.00495	0.01049	0.12108	0.00337	0.03821	0.05376	0.00485	0.00327	0.00000	0.06970	0.05336	0.00168	0.00000	0.00069
N. greenwoodi Luanso (n=10)	0.06666***	0.14060***	0.00110	0.05276***	0.08452***	0.04529**	—	0.00218	0.03029	0.00059	0.00099	0.00059	0.00010	0.00059	0.00000	0.00119	0.00000	0.00020	0.00000	0.00069
N. greenwoodi Marumbi (n=8)	0.02073	0.08596***	0.01569	0.01609	0.05872***	0.04550*	0.04883**	—	0.14583	0.07702	0.05792	0.00317	0.01505	0.00010	0.00000	0.00238	0.00000	0.00040	0.00000	0.00000
P. macrocephala Hippo (n=5)	0.05641*	0.08087*	0.02651	0.03100	0.02615	0.03653	0.04705*	0.02463	—	0.83635	0.05415	0.05633	0.18780	0.06049	0.02505	0.01663	0.17642	0.13840	0.01406	0.00950
P. macrocephala Python (n=10)	0.06154***	0.08228***	0.02739*	0.05959***	0.06911***	0.04232**	0.04488***	0.01965	0.000	—	0.82833	0.02614	0.03911	0.00228	0.00000	0.00970	0.00347	0.00307	0.00000	0.00188
P. macrocephala Luanso (n=6)	0.04575**	0.09651**	0.04053*	0.07678***	0.04026*	0.05730*	0.06116***	0.02877	0.04251	0.000	—	0.13078	0.08544	0.02752	0.00267	0.00505	0.02000	0.00614	0.00891	0.00475
P. macrocephala Marumbi (n=8)	0.09862***	0.09305**	0.03730**	0.07682***	0.07797***	0.03485	0.07940***	0.06010**	0.04679	0.03045*	0.02507	—	0.05504	0.00069	0.00000	0.03455	0.00069	0.00020	0.00020	0.00347
P. nyererei Hippo (n=6)	0.04895*	0.08214**	0.05223**	0.07282***	0.07659***	0.06717**	0.09524***	0.04781*	0.02091	0.02963*	0.03042	0.03847	—	0.11395	0.00327	0.22820	0.22899	0.16226	0.03485	0.00822
P. nyererei Python (n=10)	0.06579***	0.10244***	0.03906**	0.07527***	0.06956***	0.04601**	0.05840***	0.06788***	0.03417	0.03993**	0.03862*	0.06435***	0.02068	—	0.00762	0.17048	0.00287	0.18038	0.00970	0.00119
P. nyererei Luanso (n=9)	0.09170***	0.12381***	0.06664***	0.10032***	0.10250***	0.07678***	0.09186***	0.08002***	0.05013*	0.07266***	0.07305**	0.10891***	0.05521**	0.03770**	—	0.11930	0.00099	0.00000	0.60380	0.00149
P. nyererei Marumbi (n=4)	0.08176**	0.15046**	0.03578	0.09126***	0.14481**	0.05493	0.09356**	0.07891**	0.06816*	0.05594**	0.10925**	0.05905*	0.02176	0.02066	0.02962	—	0.05603	0.04029	0.12662	0.08544
P. pundamilia Hippo (n=6)	0.12306***	0.07042	0.11837***	0.12519***	0.11689***	0.06297	0.15015***	0.10979***	0.04741	0.08117**	0.07313*	0.11126***	0.03269	0.09029**	0.10675***	0.08381	—	0.00089	0.01327	0.00307
P. pundamilia Python (n=8)	0.10903***	0.11639***	0.06109***	0.10176***	0.07962***	0.05621**	0.08671***	0.07914***	0.02554	0.03921**	0.06061**	0.07286***	0.01749	0.01334	0.08201***	0.04066*	0.10141***	—	0.01069	0.00000
P. pundamilia Luanso (n=9)	0.09174***	0.12378***	0.07435***	0.10986***	0.08532***	0.08626***	0.11091***	0.08683***	0.06303*	0.08641***	0.06953**	0.10363***	0.04109*	0.04731**	0.000	0.02992	0.07257*	0.04838*	—	0.00248
P. pundamilia Marumbi (n=4)	0.20515**	0.21315**	0.13678***	0.18959**	0.24918***	0.16335***	0.23585***	0.16471***	0.19450**	0.14989**	0.22103**	0.13722**	0.09143**	0.17862**	0.15606**	0.07278	0.16838**	0.18022***	0.12722**	—



(a)

(b)

Figure 1: Species and sampling sites. (a) Lake Victoria, the Mwanza Gulf, and the four sampling localities, (1) Hippo Island, (2) Python Islands, (3) Luanso Island, and (4) Marumbi Island (Tanzania). (b) A neighbour joining tree for the five species investigated. Allele frequencies were estimated from AFLP data in AFLP-Surv and Reynolds genetic distance was calculated with 100 bootstrap replicates.

threshold was set to 5, relative peak height threshold set to 5% and the confidence level set to 95%. Maximum bin width was set to one. Loci that were fixed for the same allele in all populations were excluded from further analysis. From 4 primer pair combinations (cat-aag, cat-acc, cta-aag, and cta-acc), we obtained 654 polymorphic loci, 176, 188, 141, and 149 loci were obtained, respectively, from the different primer pairs. 19% of traces (randomly chosen across all plates) were repeated from restriction-ligation onwards and scored blind; mean repeatability was 88%. In order to obtain population genetic parameter estimates, the assumption was made that all populations were in Hardy-Weinberg equilibrium, an assumption supported by codominant marker studies conducted in parallel for many of the same populations [20, 30].

2.5. Phylogenetic Analysis. Allele frequencies were estimated from AFLP data assuming Hardy-Weinberg equilibrium,

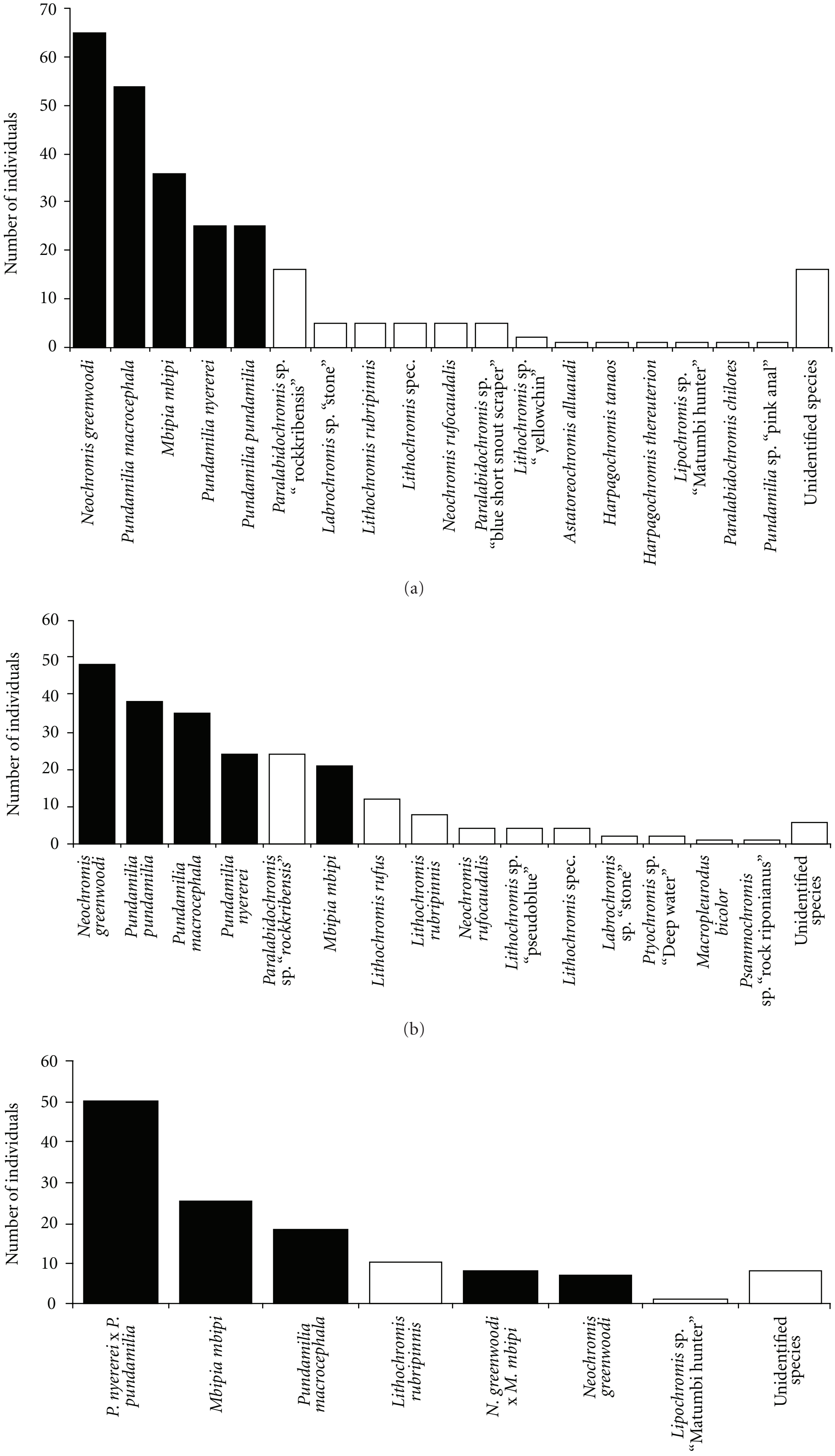
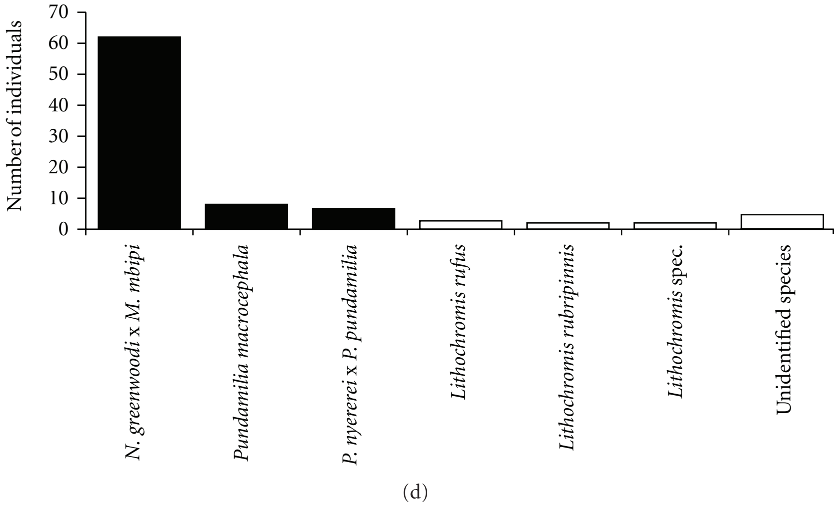


Figure 2: Continued.



(d)

Figure 2: Species-abundance composition of the communities of rock-dwelling cichlids at the 4 study islands, from top to bottom: Hippo Island, Python Island, Luanso Island, and Marumbi Island. Black bars are the taxa studied in this paper. Cumulatively, they account for between 71% and 86% of the cichlids in each community.

using a Bayesian method with nonuniform prior distribution in AFLP-Surv [31]. Reynolds et al. [32] genetic distance was calculated between (i) populations and (ii) species, with 100 bootstrap replicates. Neighbour joining trees were constructed with a randomised input order in PHYLIP [33] and consensus trees built, which were visualised in FigTree v1.3.1. To visualize conflicting phylogenetic signal among individuals, such as would be introduced by introgressive hybridization, the Nei and Li distance matrix of individuals was used to create a phylogenetic network based on the neighbour-net algorithm [34] as implemented in SplitsTree [35]. We included in the latter analysis 12 individuals of cichlid fish from Lake Edward (*Astatotilapia elegans* ($n = 2$), *A. aeneocolor* ($n = 1$), *A. sp.* "red chest" ($n = 2$), *A. sp.* "orange shoulder" ($n = 1$), *Enterochromis* sp. ($n = 1$)) and Lake Saka (Edward basin; $n = 5$) as a control for the interpretation of the distribution of phylogenetic conflict. Cichlids in the Lake Edward basin have been isolated from those in the Lake Victoria basin for at least several thousand years [36].

2.6. Population Genetic Analysis. Genetic differentiation of allopatric populations and of sympatric and allopatric species was estimated calculating the genetic distances as an equivalent of pairwise F_{ST} using the pairwise genetic distance option in Arlequin 2.0 [37] that counts the number of different alleles between haplotypes and results in weighted F_{ST} statistics over all loci [38, 39]. Throughout, we use F_{ST} as a term for this equivalent distance. We used 10 000 permutations to acquire P values. To test whether genetic differentiation of populations rather depended on morphological similarity or on geographical overlap, five groups of population pairs were made: (1) allopatric populations of the same morphological species, (2) sympatric populations of morphologically similar taxa that also turned out to be phylogenetic sister taxa (Figure 1), (3) allopatric populations of morphologically similar and phylogenetic sister taxa, (4) sympatric populations of morphologically dissimilar phylogenetic nonsister taxa, and (5) allopatric populations of morphologically dissimilar phylogenetic nonsister taxa. These groups correspond to those analyzed by Rico et al. [13] in Lake Malawi. Fixation indices were compared between these groups using a nonparametric Kruskal-Wallis test, because a normal distribution of F_{ST}-equivalent values could not be assumed a priori and group sizes and variances were heterogeneous. To determine which groups differed significantly, a Mann-Whitney test was then calculated in SPSS with the Holm Sequential Bonferroni posthoc test [40] to account for multiple testing. Finally, we calculated a spatial autocorrelation coefficient for the total dataset combined, across 5 distance classes using GenAlEx [41].

3. Results

The species tree (Figure 1(b)) estimated from allele frequencies across all four populations of each species faithfully recovered the morphologically based classifications [27]: The morphologically similar *Neochromis greenwoodi* and *Mbipia mbipi* were phylogenetic sister taxa with high bootstrap support, and so were the morphologically similar *Pundamilia pundamilia* and *P. nyererei*, whereas *P. macrocephala* was more distantly related to the others, but somewhat closer to the other *Pundamilia* species. Since morphology and molecular markers agree remarkably well on the relationships between our five study species, we refer to them henceforth as sister and nonsister species or taxa. Note that our use of the words sister species and sister taxa is meant to reflect that these species are the most closely related among the species we sampled. We make no claim that these would remain each other's closest relatives if taxon sampling was increased within and particularly beyond our four islands. Morphological data predict that *Neochromis* *greenwoodi* and *Mbipia mbipi* indeed both have other closer relatives elsewhere in the lake.

Spatial autocorrelation analysis (Figure 3) demonstrated a positive autocorrelation coefficient outside the 5% and 95% confidence limits among individuals within about 4 km range of one another. When the analysis was repeated for each species individually, the same pattern held for all except *P. macrocephala*. Our islands are on average 9.2 km apart. Because of this, and since there was no gradual isolation by distance effect, our distinction between "sympatric" and "allopatric", where the latter refer to populations living at different locations, seems appropriate.

Our collecting effort at each of the four islands revealed that the five study species were the five numerically dominant species at each of the islands, except at Python Islands where *Paralabidochromis* sp. "rockkribensis" was more abundant than *Mbipia mbipi* (Figure 2). Together the five species accounted for between 71% and 87% of all cichlids in these assemblages, and they were the only species that occurred at each of the islands, rendering them suitable for a full factorial sampling design.

The equivalent of population-pairwise F_{ST} estimates for AFLP's ranged from <0.01 to 0.25 (Table 1). Negative F_{ST} values (3 out of 190 estimates) were set to zero. Twenty-six of the 190 pairwise fixation indices were below 0.03, with three exceptions to these were not significantly different from zero. The category of pairs with values lower than 0.03 consisted mainly of populations of the same species or of sister species, with 8 pairs of allopatric conspecific populations (8 out of 30 pairs of allopatric populations, 27%), 5 pairs of allopatric sister species (5 out of 24 pairs, 21%) and 4 pairs of sympatric sister species (4 out of 8 pairs, 50%). Fixation indices for nonsister species were less likely to be this low; that is, only 2 of 32 (6%) pairs of sympatric populations of nonsister species and 7 of 96 (7%) pairs of allopatric populations of nonsister species fell into the $F_{\mathrm{ST}} < 0.03$ category. Of the F_{ST} estimates that exceeded 0.03, fourteen were not significant either. These were mostly associated with pairs in which at least one population had a sample size below six. For further analyses, we excluded populations of which we had less than six complete multilocus genotypes (3 populations were thereby excluded).

We then had 136 estimates of pairwise fixation indices left. Among these were 21 allopatric pairs of conspecific populations, and 17 of them were significantly differentiated, implying that many of these island populations are geographically at least partially isolated. On the other hand, with one exception, sympatric populations of sister taxa were not significantly differentiated, whereas all 22 sympatric populations of nonsister taxa were.

The Kruskal-Wallis test revealed that there were differences between the five population groupings ($P < 0.001$). Mann-Whitney tests revealed that sympatric populations of sister taxa were significantly less differentiated than any other type of populations including allopatric populations of conspecifics (Figure 4, Table 2). Sympatric populations of nonsister taxa on the other hand, were significantly more differentiated than sympatric populations of sister taxa, and very similar in their F_{ST} to allopatric populations of both sister and nonsister species. Allopatric populations of conspecifics were slightly, but not significantly less differentiated than allopatric populations of sister species, but they were significantly less differentiated than allopatric populations of nonsister species. Finally, sister species, when sampled in sympatry, were significantly less differentiated than when sampled in allopatry.

The individual-based neighbour net (Figure 5(a)) revealed a large amount of phylogenetic conflict about the placement of individuals of the five species and 20 populations of Lake Victoria cichlids, whereas there was somewhat less conflict about the placement of 12 individuals of Lake Edward basin cichlids relative to the taxa from Lake Victoria. The population tree (Figure 5(b)) was much better sorted

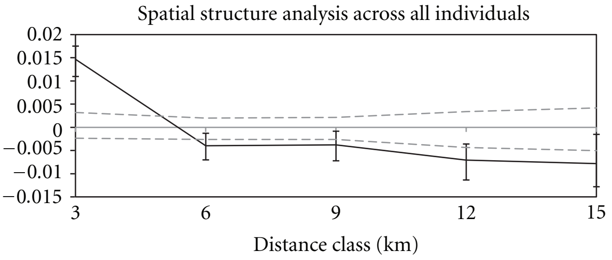


Figure 3: Spatial structure analysis reveals a positive autocorrelation coefficient outside the 5% and 95% confidence limits (dashed lines) among individuals within about 4 km of one another, but no isolation by distance beyond this.

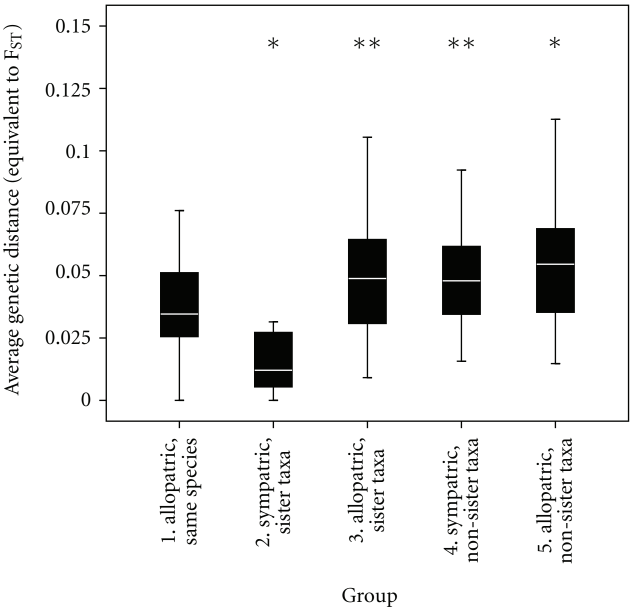


Figure 4: F_{ST} equivalent estimates for pairwise population comparisons falling into one of five different groupings: populations of the same species (always allopatric), morphologically similar taxa (*M. mbipi* and *N. greenwoodi* and *P. pundamilia* and *P. nyererei*) in sympatry and in allopatry, and nonsimilar taxa in sympatry and in allopatry. A Holm Sequential Bonferoni posteriori test was used to obtain P values. *Represents a significant difference against group 1, **represents a significant difference against group 2.

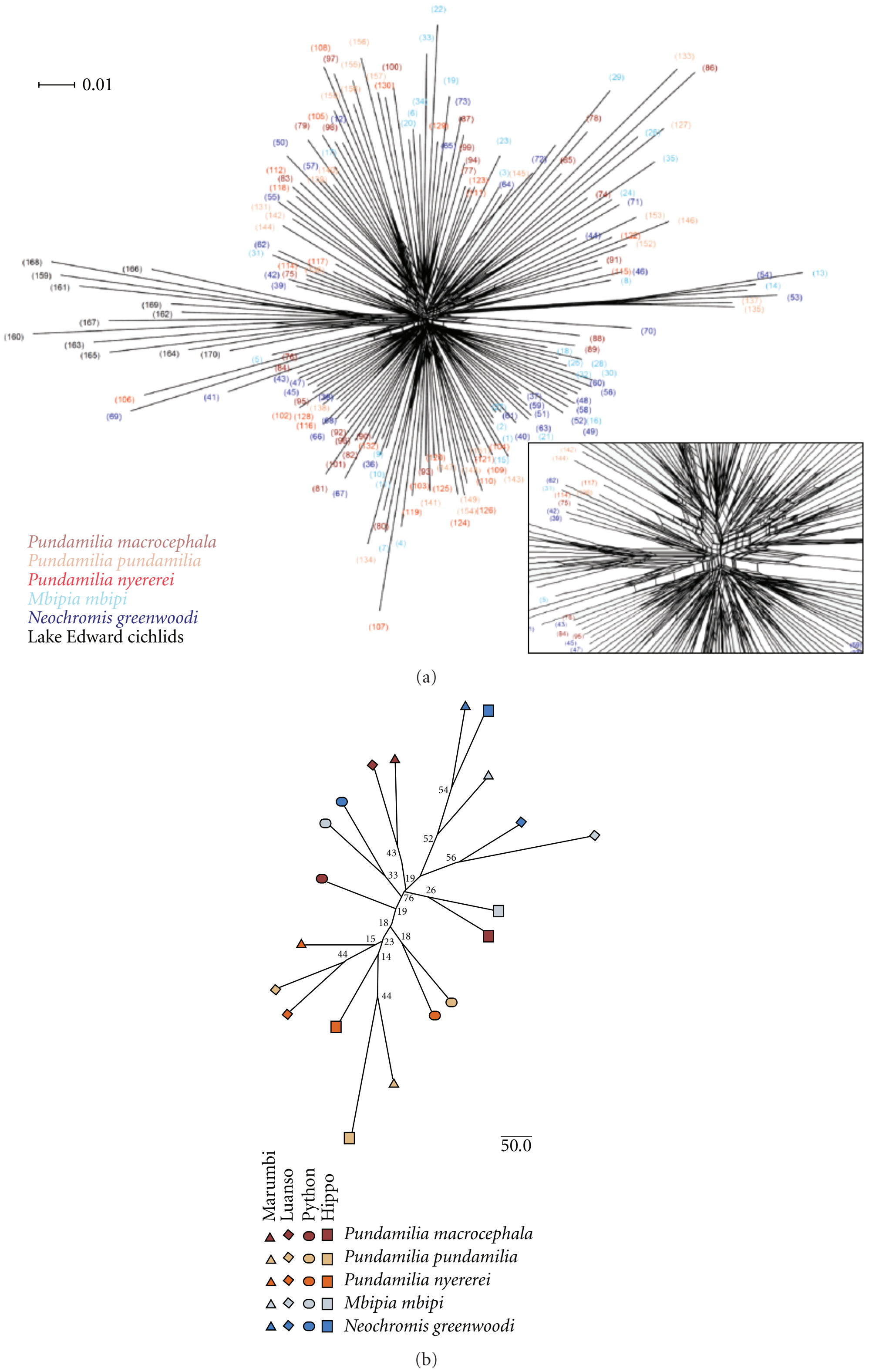


FIGURE 5: Individual-based and population trees. (a) An AFLP neighbor network based on Nei and Li distances. Samples are sorted and colour-coded by species. Conflicting phylogenetic signal in the center magnified bottom right. (b) A Neighbour joining tree for the 20 populations investigated. Allele frequencies were estimated from AFLP data in AFLP-Surv, and Reynolds genetic distance was calculated with 100 bootstrap replicates. The 4 different shapes indicate the 4 islands. The five different colours indicate the 5 species.

Table 2: Mann-Whitney test comparing F_{ST}-equivalent estimates between populations grouped by geography and relatedness. P values are two tailed. Posteriori test: Holm Sequential Bonferroni (with 7 comparisons). NT: tests not performed because they are not testing predictions of our hypotheses. Results are graphically represented in Figure 4.

Groups	1	2	3	4	5
1 = Allopatric, same species	—				
2 = Sympatric, sister species	**0.008**	—			
3 = Allopatric, sister species	0.147	**0.004**	—		
4 = Sympatric, non-sister species	NT	**<0.001**	NT	—	
5 = Allopatric, non-sister species	**0.003**	NT	0.505	0.584	—

than the individual-based tree but reflected the conflicting signal of phylogenetic relationship (taken here as the species tree of Figure 1) on the one hand, and local gene flow between sympatric species on the other hand. The two pairs of sister species were reciprocally monophyletic and *P. macrocephala* tended to be more distantly related to either, with signals of introgressive hybridization with *Neochromis/Mbipia* at Hippo and Python Islands. Interestingly, the abundance of *P. macrocephala* relative to *Neochromis* and *Mbipia* is much greater at these two islands than at the other two (Figure 2). Sympatric populations of sister species were in four cases each other's closest relatives, *P. pundamilia* and *P. nyererei* at Python and Luanso Islands, and *N. greenwoodi* and *M. mbipi* at the same two island locations.

4. Discussion

We studied five different Lake Victoria cichlid species that live on spatially isolated patches of habitat, around rocky islands. Based on morphological data [27] this set of species contains two pairs of putative sister taxa, *Pundamilia pundamilia* and *P. nyererei*, *Neochromis greenwoodi* and *Mbipia mbipi*, and one species, *Pundamilia macrocephala* that is slightly more distantly related to either. We found this morphological hypothesis to be faithfully recovered by a species tree based on 654 AFLP loci. It was also quite well recovered by a population tree based on the same loci, but there were strong signals of interspecific gene flow in sympatry, particularly between closely related species. An individual-based neighbor net indeed revealed large amounts of phylogenetic conflict, suggestive of considerable introgressive hybridization.

Within each local species assemblage, we find good correspondence between relatedness (morphological expectations and AFLP species tree) and the AFLP-derived fixation indices. Our sample sizes per population were small, and even though this is partly compensated for by our use of a large number of loci, caution is warranted with regard to taking individual pairwise genetic distance values at face value. On the other hand, all our hypothesis tests are based on multiple replicates of pairwise comparisons from four different islands, making our main results robust to sampling error. Genetic distance between sister taxa were always lower than those between nonsister taxa, where the latter were always significant. This was true at all islands, hence in clearer waters in the northern Mwanza Gulf as much as in the very turbid waters in the South. Genetic distance between *Pundamilia macrocephala* and the species of either of the other two species pairs varied but was sometimes low and nonsignificant. Hence, at the scale of local island assemblages, *P. pundamilia* and *P. nyererei* and also *N. greenwoodi* and *M. mbipi* are indeed consistently two different pairs of population genomic sister species, and *P. macrocephala* has somewhat varying affinities to either of these taxon pairs. To the best of our knowledge, this is the first molecular genetic support for any above-species level taxonomical groupings among Lake Victoria cichlid fish. The remarkably well resolved species tree that recovers morphology-based hypotheses about relatedness among species is a definitive first in phylogenetic analyses of Lake Victoria cichlids. We suspect that our full factorial sampling design, averaging out over four islands the locally variable effects of heterospecific gene flow, may have been key to this.

Adding geographical proximity complicates the picture. Allopatric populations of the same phenotypically defined species tend to be more strongly genetically differentiated than sympatric populations of phenotypically divergent sister species. This was observed previously for the species pair *P. pundamilia* and *P. nyererei* using microsatellite markers [20], but here, we show that the same is true for a second pair of species, *Neochromis greenwoodi* and *Mbipia mbipi,* and is also often true for *Pundamilia macrocephala* against either of these other species. On the other hand, morphologically quite different species, that is, *Pundamilia pundamilia/nyererei* versus *Neochromis/Mbipia,* are genetically well differentiated independent of whether the differentiation is measured among sympatric or among allopatric populations.

Our results differ from those obtained for a similar set of Lake Malawi rock-dwelling cichlid fish by Rico et al. [13]. Their comparisons revealed weaker genetic differentiation among allopatric populations of putative conspecifics than among sympatric populations of closely related species. Further, they found no indication that sympatric populations of closely related species were any less differentiated than allopatric populations of closely related species. From this, they concluded that there was no significant gene flow among sympatric species now or in the recent past. Rico et al. [13] proposed their analysis as a test of the hypothesis of parallel sympatric speciation within locations, which they could clearly reject with their data for the species they sampled.

By the same token, our analysis of Lake Victoria cichlids would be consistent with the hypothesis of parallel sympatric speciation on our four islands.

However, although we think parallel sympatric speciation is a possibility, perhaps a more likely explanation of our findings is that the rate of gene flow among sister species or morphologically similar species in sympatry exceeds that of gene flow between these species when they live at different islands, but also that between conspecific populations living at different islands. This situation may not be unlike that in ground finches on the Galapagos islands [5, 42], where introgressive hybridization among sympatric species is common and affects evolutionary trajectories. In either case, however, we conclude that considerable phenotypic differentiation can be maintained in Lake Victoria cichlids against a very significant amount of heterospecific gene flow. This also suggests that fairly large parts of the genome might be exchanged among species without affecting morphology in a dramatic way. Conversely, and perhaps more surprisingly, phenotypic resemblance between conspecific island populations can be maintained despite fairly little gene flow among these conspecific populations, and in fact, less than the gene flow these populations receive from sympatric heterospecifics. Speciation in Lake Victoria cichlids does not seem to happen simply as an idiosyncratic byproduct of geographical isolation, and it may indeed not even require geographical isolation on separate islands. If parallel speciation within locations was indeed explaining our observations, the phenotypic resemblance among similar species evolved in parallel on different islands would be stunning.

There are several possible alternative explanations for the differences between our study and that of Rico et al. [13]. The differences might be due to the different age of these radiations and the associated speciation events. The radiation of Lake Victoria cichlids into the current species (which is not the same as the age of the allelic variants segregating in the radiation) [36, 43] is probably one order of magnitude younger than that of the Lake Malawi Mmbuna [14, 44]. Perhaps speciation is no longer very frequent among the Lake Malawi Mmbuna, in which case reproductive isolation between species may have become stronger through time. Alternatively, the differences might be due to the particular set of Mmbuna species or the set of microsatellite loci that were investigated by Rico et al. [13].

Our data are also in contrast with those of Samonte et al. [18] who did not find any genetic structure among four other morphologically defined species of Lake Victoria cichlids, using 11 genetic markers. This difference may either be explained by the choice of species, or (perhaps more likely), by the number of genetic markers. Given the very short time since speciation began in Lake Victoria, and the evidence for interspecific hybridization, lineage sorting is expected to be highly incomplete and genetic differentiation among sympatric species may only reveal itself when a larger number of loci is at hand, for example, [25].

The intensity of gene flow between the species that we studied has almost certainly been affected by recent anthropogenic eutrophication, particularly in the South of our sampling region (i.e., Luanso and Marumbi islands), but even at Hippo Island water clarity has decreased. This does not invalidate any of the above discussion points. First, even though sympatric sister taxa tend to be genetically less differentiated at the southern than at the northern locations, we find even at the northern locations that sympatric sister taxa are less strongly differentiated than allopatric conspecifics and allopatric nonsister species. Second, remarkable gradients in water clarity have existed in Lake Victoria prior to anthropogenic eutrophication even though the turbid zone has recently expanded considerably into the open lake [19]. Third, paleoecological evidence suggests that since its last desiccation, Lake Victoria has undergone cycles of severe anthropogenic eutrophication and recovery on the order of thousands of years [45]. For all these reasons, we think that some gene flow between species in sympatry is most likely not just a very recent phenomenon but that the rate of hybridization has recently increased in the more turbid parts of the lake due to man-made eutrophication. However, here for the first time, we show that this anthropogenically increased rate of hybridization does not affect all sympatric species evenly, but it really affects predominantly closely related species. This has important implications for the evolution of reproductive isolation between species and its dimensionality. It is also predicted by the hypothesis of speciation reversal due to weakening of divergent selection and reproductive isolation as a consequence of habitat homogenization [46] and offers an evolutionary mechanistic explanation for the observation that the species poor assemblages in turbid areas of the lake are characterized by just one or two species in each of a few morphologically distinct genera [27].

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Research Article

A Molecular Perspective on Systematics, Taxonomy and Classification Amazonian Discus Fishes of the Genus *Symphysodon*

Manuella Villar Amado,[1,2] Izeni P. Farias,[1] and Tomas Hrbek[1,3]

[1] *Laboratório de Evolução e Genética Animal, Departamento de Biologia, Universidade Federal do Amazonas, Avenida Rodrigo Octávio Jordão Ramos, 3000, 69077-000 Manaus, AM, Brazil*
[2] *Instituto Federal de Educação, Ciência e Tecnologia do Espírito Santo, Unidade Vitória, Avenida Vitória, 1729, 29040-780 Vitoria, ES, Brazil*
[3] *Biology Department, University of Puerto Rico—Rio Piedras, 00931 San Juan, PR, Puerto Rico*

Correspondence should be addressed to Tomas Hrbek, hrbek@evoamazon.net

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With the goal of contributing to the taxonomy and systematics of the Neotropical cichlid fishes of the genus *Symphysodon*, we analyzed 336 individuals from 24 localities throughout the entire distributional range of the genus. We analyzed variation at 13 nuclear microsatellite markers, and subjected the data to Bayesian analysis of genetic structure. The results indicate that *Symphysodon* is composed of four genetic groups: group PURPLE—phenotype Heckel and abacaxi; group GREEN—phenotype green; group RED—phenotype blue and brown; and group PINK—populations of Xingú and Cametá. Although the phenotypes blue and brown are predominantly biological group RED, they also have substantial contributions from other biological groups, and the patterns of admixture of the two phenotypes are different. The two phenotypes are further characterized by distinct and divergent mtDNA haplotype groups, and show differences in mean habitat use measured as pH and conductivity. Differences in mean habitat use is also observed between most other biological groups. We therefore conclude that *Symphysodon* comprises five evolutionary significant units: *Symphysodon discus* (Heckel and abacaxi phenotypes), *S. aequifasciatus* (brown phenotype), *S. tarzoo* (green phenotype), *Symphysodon* sp. 1 (blue phenotype) and *Symphysodon* sp. 2 (Xingú group).

1. Introduction

Tropical regions contain many more species than do temperate and polar regions; however, explanations as to why remain unclear; for example, see [1, 2]. The Amazon Basin is particularly species rich and harbors arguably the world's greatest terrestrial [3, 4] and freshwater [5] biodiversity. Numerous potential processes generating this diversity within the Amazon basin have been proposed; for example, see [6–8]. Testing of processes that have generated Amazonian biodiversity depends on solid taxonomy, since species are the operational units in all studies of biodiversity; for example, see [9]. The taxonomy of many Amazonian groups is still poorly known [3, 5] and at least in fishes a number of instances of haplotype sharing between closely related species have been reported; for example, see [10–14]. This makes it difficult to apply the phylogenetic species concept in species discovery and diagnosis. The delimitation of species of the genus *Symphysodon* has also been problematic in part due to sharing of mitochondrial DNA haplotypes between species [15–17] and a complete lack of resolution with nuclear DNA haplotypes [15, 17].

The discus fishes (genus *Symphysodon*) have a natural distribution is the Amazon basin. Traditionally, five principal phenotypic, largely allopatrically distributed groups are recognized in popular literature [18, 19]. These groups are (1) green phenotype—found in the western Amazon basin, (2) blue phenotype—found in the central Amazon basin, (3) brown phenotype—found in the eastern Amazon basin, (4) Heckel phenotype—found in the Negro and Trombetas River basins, and (5) abacaxi phenotype—found in the Abacaxis River, a blackwater tributary of the lower Madeira River.

Since the description of the type species and the genus by Heckel in 1840, there has been taxonomic uncertainty and confusing classification related to the genus *Symphysodon*. Considering descriptions and taxonomic revisions until the year 2006, two species were recognized in scientific literature [20, 21]: *Symphysodon discus* Heckel, 1840 and *Symphysodon aequifasciatus* Pellegrin, 1904, and four subspecies in popular literature: *S. discus willischwartzi* Burgess, 1981 (phenotype abacaxi), *S. discus tarzoo* Lyons, 1959 (phenotype green), *S. aequifasciatus haraldi* Schultz, 1960 (phenotype blue) and *S. aequifasciatus axelrodi* Schultz, 1960 (phenotype brown), with the nominal sub-species *S. discus discus* Heckel, 1840 (phenotype Heckel), and *S. aequifasciatus aequifasciatus* Pellegrin, 1904 (phenotype green), being restricted to just one major phenotype. Recently, Ready et al. [17] proposed the existence of three species: *S. discus* (phenotype Heckel and abacaxi), *S. aequifasciatus* (phenotype blue and brown) and *S. tarzoo* (green phenotype). Bleher et al. [16] also argued for the existence of three species: *S. discus* (phenotype Heckel and abacaxi), *S. aequifasciatus* (phenotype green) and *S. haraldi* (phenotype blue and brown), with *S. aequifasciatus* and *S. haraldi* occurring allopatrically but throughout the Amazon basin (i.e., the species are not restricted to the western, and central+eastern Amazon basin). Farias and Hrbek [15] on the other hand argued that the genus *Symphysodon* is probably a biological complex in the process of speciation. Farias and Hrbek [15] also reported the existence of an additional deeply divergent mitochondrial lineage from the Xingú River basin.

All three studies found individuals of the green phenotype to form a monophyletic mtDNA group. Farias and Hrbek [15] also observed monophyly of the blue phenotype and the Xingú lineage. All three studies [15–17] observed haplotype sharing between the Heckel, abacaxi, and brown phenotypes, with Bleher et al. [16] considering the brown phenotype individuals to be blue phenotype individuals introgressed with Heckel phenotype mtDNA.

Inclusion of nuclear DNA sequence data did not identify monophyletic groups or clarify taxonomy of *Symphysodon*. Farias and Hrbek [15] analyzed the third exon the recombination activation gene one (RAG1) observing extensive haplotype sharing among all phenotypes. Ready et al. [17] included the Rhodopsin gene in their analysis; however, it showed no sequence variation within *Symphysodon*.

The two published isozyme studies of *Symphysodon* [22, 23] could not find any diagnostic marker that would separate *S. discus* and *S. aequifasciatus*, and Kokoscha and Greven [23] even observed that among-population divergences within *S. aequifasciatus* were larger than the interspecific genetic divergence of these populations and *S. discus*. Two mtDNA sequence studies by Zhang et al. [24, 25] of aquarium material also reported that interspecific divergence was smaller than among-population divergences within *S. aequifasciatus*. Results from chromosomal studies of Gross and collaborators [26, 27] have reported extensive karyotypic variation within and among sampling localities but have found no consistent karyotype differences between different species and populations of *Symphysodon*.

In spite all of these studies, the taxonomy of this group remains elusive. Genetic characterization of *Symphysodon* and identification of biological populations are essential for the understanding of evolutionary processes operating on the genus *Symphysodon* and for delimitation of evolutionary species. Neutral molecular markers with high mutation rates have the greatest ability to record signatures of recent evolutionary events.

Microsatellite loci have a mutation rate estimated at 2.5×10^{-3} in humans [28, 29] to 5.6×10^{-4} [30] mutations per generation with similar values observed in mice [31], and are commonly used for intraspecific population studies, for example, [32–35]. Since microsatellite flanking regions are generally conserved among closely related species, they are also often used for interspecific studies and studies of species complexes, for example, see [36–41]. Microsatellite markers, thus, lend themselves well to delimiting taxonomic boundaries and identifying cases of hybridization [42–44].

The aim of the present study was to use microsatellite markers to genetically characterize phenotypic variants of *Symphysodon* sampled throughout their area of natural distribution and to investigate association between genetic variants, geographic distribution, and described species/subspecies. We also use the proxy variables of pH and conductivity to test for differences in mean habitats occupied by the different phenotypes and groups. Neither pH nor conductivity are necessarily the primary agents causing ecological separation, but they are likely to reflect differences in habitats occupied by the different phenotypes as correlates of other important environmental variables or differences in the geological history of the areas occupied by species of *Symphysodon*.

2. Material and Methods

2.1. Samples. A total of 336 individuals were sampled from 24 localities (Figure 1) throughout the known geographic distribution of the genus *Symphysodon*. All five principal phenotypes (Heckel, abacaxi, brown, green, and blue) were sampled, with an average number of 13 individuals per sampled locality. Individuals were assigned to phenotype following Farias and Hrbek [15]. Fishes were collected by cutting and submerging branches in appropriate habitats on the margins of lakes and small rivers for approximately a week to allow sufficient time for individuals to colonize this habitat (popularly known as "galhada"). After the fish colonized this habitat, they were collected with nets. Samples were also obtained from local ornamental fishermen. A small sample of tissue from the caudal peduncle or the left pectoral fin was removed and stored in 96% ethanol until processing in the laboratory, with remainder of the specimen preserved in 10% formalin, and after fixation transferred to 70% ethanol. Tissue and specimen samples are stored in the tissue collection of the Laboratory of Evolution and Animal Genetics (LEGAL) of the Federal University of Amazonas, Manaus, Brazil.

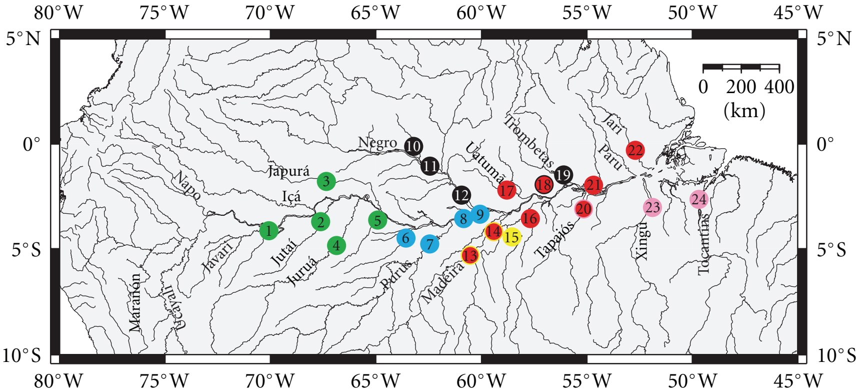


Figure 1: Distribution and collecting areas of phenotypes of the genus *Symphysodon*. Numbers represent: (1) Tabatinga (Calderão River), (2) lower Jutaí River, (3) Lake Santa Maria (Japurá River), (4) Bauana (Juruá River), (5) Igarapé Bowona (Tefé River), (6) Lake Coari (Coari River), (7) Lake Castanho (Purus River), (8) Lake Manacapuru (Manacapuru River), (9) Iranduba/Mamuri (Solimões River), (10) lower Demini River, (11) Igarapé Bui-Bui (Negro River), (12) Novo Airão (Negro River), (13) Novo Aripuanã-Acari (Madeira River), (14) Nova Olinda do Norte (Madeira River), (15) central Abacaxis River, (16) Maues (Maués River), (17) Itapiranga (Uatumã River), (18) Nhamundá (Nhamundá River), (19) Porto Trombetas (Trombetas River), (20) Belterra (Tapajós River), (21) Lake Grande (Amazonas River), (22) Igarapé Arapiranga (Jari River), (23) Vitória do Xingu (Xingu River), and (24) Cametá (Tocantins River). Colors correspond to the phenotypes analyzed in this study: green = green phenotype; blue = blue phenotype; black = Heckel phenotype; yellow = abacaxi phenotype; red = brown phenotype; pink = Xingu clade. Localities 13/14 and 18 contained the "abacaxi" and "brown" phenotypes and the "Heckel" and "brown" phenotypes, respectively.

2.2. DNA Extraction and Amplification. Total DNA was extracted from tissue samples using the phenol/chlorophorm protocol [45]. For some samples, the Genomic Prep Cells and Tissue DNA Isolation kit (GE Healthcare) was used.

To characterize *Symphysodon* individuals, we used 13 microsatellite pairs developed by Amado et al. [46]. Genotyping was done according to the economical method of Schuelke [47] that uses a 5′ tailed amplification primer, and then a third fluorescently labeled primer in the genotyping reaction. This way just one labeled primer can be used to fluorescently label multiple microsatellite loci.

PCR reactions for all primer pairs were carried out in a final volume of 10 μL containing 4.5 μL of ddH$_2$O, 0.7 μL of MgCl$_2$ (25 mM), 0.8 μL of dNTPs (10 mM), 1.0 μL of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl), 0.5 μL of forward primer with M13(-21) 5′ tail (2.0 μM), 1.0 μL of reverse primer (2.0 μM), 0.5 μL of fluorescently-labeled M13(-21) primer (2.0 μM), 0.2 μL of Taq DNA Polymerase (5 U/μL), and 1 μL of DNA (concentration varied between 50 ng and 100 ng). PCR reactions were performed in two stages, an amplification stage, and labeling stage. For the microsatellite loci Sd04 and Sd05 the amplification reaction consisted of denaturation at 94°C for 60 seconds, followed by of 25 cycles of denaturation at 93°C for 5 seconds, primer annealing at 65°C for 5 seconds, and primer extension at 68°C for 10 seconds. For the microsatellite loci Sd08 and Sd10, the amplification reaction consisted of denaturation at 94°C for 60 seconds, followed by of 35 cycles of denaturation at 93°C for 20 seconds, primer annealing at 65°C for 20 seconds, and primer extension at 68°C for 30 seconds. For the microsatellite loci Sd11, Sd12, Sd14, Sd15, Sd22, Sd23, Sd25, Sd27, and Sd30 the amplification reaction consisted of denaturation at 94°C for 60 seconds, followed by of 35 cycles of denaturation at 93°C for 20 seconds, primer annealing at 55°C for 20 seconds, and primer extension at 68°C for 30 seconds. The PCR labeling stage consisted of 25 cycles of denaturation at 94°C for 5 seconds, primer annealing at 53°C for 10 seconds, and primer extension at 68°C for 30 seconds, followed by a final extension for 20 minutes at 68°C. Subsequent to the genotyping reaction, the PCR product was diluted 1 : 10 to 1 : 50 depending on the microsatellite used, and 1 μL of the diluted product was resuspended in 9 μL Hi-Di formamide/ET 400 size standard mix (GE Healthcare) and resolved on a MegaBace automatic sequencer (GE Healthcare). The programs Genetic Profiler and Fragment Profiler were used to extract raw fragment data and infer fragment sizes.

2.3. Statistical Analyses. The programs GenAlEx 6.41 [48] and ARLEQUIN version 3.11 [49] were used for the calculation of allelic frequencies and number of alleles, observed (H_O) and expected (H_E) heterozygosities, linkage disequilibrium between pairs of loci, and to test for Hardy-Weinberg equilibrium within sampling localities. In cases of multiple comparisons, significance was adjusted using the serial Bonferroni method proposed by Rice [50].

To estimate the degree of genetic differentiation between sampling localities and phenotypes, we calculated F_{ST}-like values [51, 52] and tested their significance via 10,000 bootstrap replicates. Hierarchical analysis of molecular variance (AMOVA) [53] was used to test four hypotheses: (1) grouping of localities into two species [21], (2) grouping of localities into three species [17], (3) grouping of localities into five phenotypes [18, 54], and (4) grouping of localities into five phenotypes and the Xingu group [15].

The data were analyzed in the program STRUCTURE version 2.3.2 [55, 56] with the goal of assigning individuals into groups, given a specific number groups (K). We used the "admixture" and "correlated-allelic-frequencies" models. Assignment space was explored with 1,000,000 MCMC chains, preceded by 100,000 MCMC chains discarded as burn-in. Each analysis was repeated ten times from a different randomly selected starting point, and independent runs summarized in the program CLUMPP 1.1.2 [57]. Results were visualized in the program DISTRUCT 1.1 [58]. The most likely number of biological groups (K) was inferred using the methodology of Evanno et al. [59] implemented in Structure Harvester 0.6.1 [60]. Since not all geographical groups and not all individuals were genetically pure, that is, composed of just one biological group, we analyzed differences in genetic composition and admixture among phenotypic groups using an MANOVA, where individual q values were the dependent and phenotypic groups the independent variables. Genetic composition of individuals was summarized in the form of principal components, and heterogeneity of genotypic composition of phenotypes was calculated from weighted eigenvalues of all contributing principal components. When the data permitted, we used a logistic regression to investigate differences in the pH and conductivity of water sampled from the habitats occupied by the different phenotypes of *Symphysodon* [16]. The distribution of phenotypes in localities was based on our understanding of the geographic distribution of phenotypes (see online supplement S1 available online at doi: 10.4061/2011/360654). All analyses were performed in the software R 2.11.1 (http://cran.r-project.org/).

To identify possible Evolutionary Significant Units (ESUs) comprising the genus *Symphysodon*, we used the methodology proposed by Crandall et al. [61]. The methodology is based on testing if geographically separated populations are genetically and ecologically exchangeable or were in the recent past.

3. Results

3.1. Genetic Diversity. The total number of alleles encountered across all the loci was 150, with an average of 11.5 ± 9.0 alleles per locus. The highest allele number was observed in the locus Sd30, and the lower number in the loci Sd10 and Sd22. Allele numbers per locus are: Sd04 (9 alleles), Sd05 (10 alleles), Sd08 (10 alleles), Sd10 (5 alleles), Sd11(10 alleles), Sd12 (8 alleles), Sd14 (12 alleles), Sd15 (25 alleles), Sd22 (5 alleles), Sd23 (8 alleles), Sd25 (4 alleles), Sd27 (8 alleles), and Sd30 (36 alleles).

Observed heterozygosity varied from 0 to 1 while expected heterozygosity varied from 0.01 to 0.96 (Table 4). Majority of the sampled localities had low observed heterozygosities (0.4–0.5), while the lowest observed heterozygosities (0.2–0.3) were observed in Demini, Novo Airão, Buibui, and Xingú. There were seven cases of Hardy-Weinberg disequilibrium. Four loci (SD04, SD08, SD11, and SD30) were at Hardy-Weinberg disequilibrium in the locality Trombetas, while the loci SD08, SD08, and SD15 were in Hardy-Weinberg disequilibrium in the localities Tabatinga, Nhamundá, and Tefé, respectively.

3.2. Genetic Differences among Species and Hierarchical Groups. Analysis of molecular variance [52] revealed that a majority of genetic variance was encountered within sampling localities rather than at higher hierarchical levels when hierarchical levels represented species or phenotypes (Table 2). All hierarchical levels were significant. Grouping reflecting the two [21] versus three [17] species classification system explained 24.80% versus 21.67% of total variance. Grouping individuals into six phenotypes [61] or five ESUs (this study) versus five phenotypic clusters [18, 54] explained more variance (22.42% and 22.41% versus 19.54%). When six phenotypic clusters or five ESUs were considered, relatively more variance was explained by among phenotype differences rather than by differences among localities within phenotypes, than in any other hierarchical grouping scheme (Table 2). In general, pairwise differences between localities (online supplement S2) were significant in majority of comparisons with the exception of most pairwise comparison involving localities of the green phenotype (Tabatinga, Jutaí, Jurua, Tefé, and Japurá) and the blue phenotype (Coari, Purus, Manacapuru, and Iranduba/ Mamuri). Pairwise differences between ESUs were also significant (online supplement S3).

3.3. Biological Groups. The most likely number of biological groups inferred in the program STRUCTURE was four (Figure 2). The majority of individuals had $q > 0.9$, that is, had >90% probability of belonging to a particular biological group, and phenotypes were composed of individuals belonging to the same biological cluster. The phenotype green was present at the localities Tabatinga, Jutaí, and Juruá, where 100% of individuals had $q > 0.9$ and in the Tefé and Japurá localities, where 88% and 84% individuals, respectively, had $q > 0.9$; fishes in all five localities belonged to the biological cluster GREEN. The phenotypes Heckel and abacaxi comprised the same biological cluster (cluster PURPLE), and 100% of individuals sampled from Buibui, Novo Airão, Demini, and Abacaxis had $q > 0.9$. In the Trombetas locality, where individuals also belong to the Heckel phenotype and are predominantly comprised of the biological cluster PURPLE, 80% of individuals had $q > 0.9$ while the remaining 20% had $q > 0.8$. In the Nhamundá and Nova Aripuana localities, some individuals had the Heckel and abacaxi phenotypes, and of these individuals 3 of 12 and 3 of 3, respectively, had $q > 0.9$. Biological cluster PINK was composed of individuals from the localities Xingú (100% of individuals with $q > 0.9$) and Cametá (53% of individuals with $q > 0.9$). This biological cluster is not recognized as a distinct taxon or phenotype in professional or popular literature, but individuals from this cluster were identified as belonging to the Xingú clade in the study of Farias and Hrbek [15]. The phenotypes blue and brown composed of individuals whole genomes were predominantly the biological cluster RED. Majority of these individuals had $q > 0.9$ although there was a relatively large number of individuals with lower q values. The low q values were largely due to sharing of genome portions principally with fishes of biological cluster PURPLE (phenotype Heckel+abacaxi) and to a lesser extent with cluster PINK (Xingú group) and cluster GREEN (phenotype green). Population level q values are summarized in Table 1 and Figure 2(b).

Table 1: Results of the program STRUCTURE assuming $K = 4$ clusters, with proportion of individuals with q values > 0.9 and 0.8 in each locality, and proportion of genomes in locality estimated in each of the four clusters. q values < 0.050 are not shown.

Locality	($q > 0.9$)	($q > 0.8$)	*Cluster* PINK (q)	*Cluster* GREEN (q)	*Cluster* RED (q)	*Cluster* PURPLE (q)
Tabatinga	1.000	1.000		0.970		
Jutai	0.900	1.000		0.971		
Jurua	1.000	1.000		0.977		
Tefe	0.880	0.960		0.943		
Japura	0.842	0.947		0.945		
Coari	0.500	0.750		0.103	0.888	
Purus	0.830	1.000			0.946	
Mamuri	0.429	0.857*		0.396	0.566	
Manacapuru	0.700*	0.900*		0.109	0.864	
Novo Airão	1.000	1.000				0.985
Bui-Bui	1.000	1.000				0.987
Demini	1.000	1.000				0.980
Nova Aripuana	0.727†	0.727†			0.604	0.373
Nova Olinda	0.700	0.900			0.916	
Abacaxis	1.000	1.000				0.977
Uatuma	0.200	0.700		0.167	0.791	
Maues	0.556*	0.778*		0.161	0.800	
Nhamunda	0.333†	0.500†		0.052	0.328	0.591
Trombetas	0.800	1.000				0.955
Tapajos	0.563	0.813	0.109		0.871	
Alenquer	0.846	0.846	0.051		0.900	
Xingu	0.950	1.000	0.975			
Jari	0.778	1.000			0.930	
Cameta	0.467	0.600	0.790	0.111	0.092	

* Includes individuals from clusters GREEN and RED; † Includes individuals from clusters RED and PURPLE.

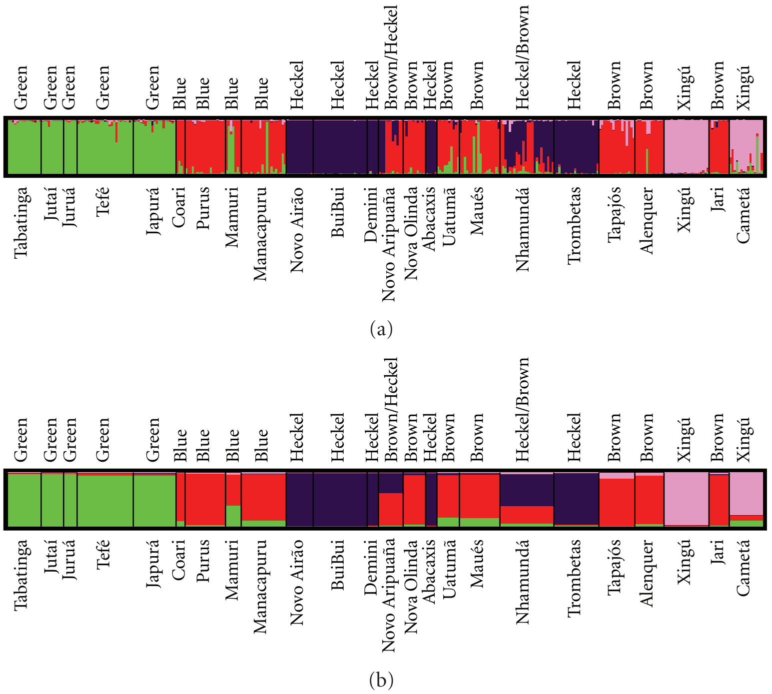
Viewed through the prism of the traditional two species taxonomy [21], all biological samples representing *Symphysodon discus* were within biological cluster PURPLE, while *Symphysodon aequifasciatus* was divided into three biological clusters (cluster RED, GREEN, and PINK). Cluster GREEN represents phenotype green found in the western Amazon basin, cluster RED represents phenotypes blue and brown from the central and eastern Amazon basin, while cluster PINK represents fishes of phenotype brown from the Brazilian Shield tributaries of the Amazon River in the eastern Amazon basin.

Although the blue group formed a well-supported mtDNA clade [15], microsatellite nDNA profile indicated that individuals of the blue and brown phenotypes predominantly belong to the biological cluster RED (most individuals have $q > 0.9$). Individuals of the blue and brown phenotypes were genetically the most admixed (Table 3), and the patterns of admixture were different between the blue and brown phenotypes, however (MANOVA of q values; Pillai's trace = 0.06324, df = 1, $P = 0.0397$).

3.4. Environmental Variables. Differences in mean habitat use between phenotypes were tested using water type [62] and water characteristics [16]. Logistic regression indicated significant differences in pH or conductivity or both in all pairwise comparisons of phenotype except the pairwise comparison of the green (*S. tarzoo*) and the blue (*Symphysodon* sp. 1) phenotype. The ranges of pH and conductivity individually or in combination did not overlap involving comparisons of the Heckel (*S. discus*) and other phenotypes.

4. Discussion

Although the east African rift lakes contain some of the most spectacular, recently evolved assemblages of cichlid fishes [63, 64], the insular environments of the Caribbean are well known for their *Anolis* and *Eleutherodactylus* radiations [65–67], and the Hawaiian islands harbor spectacular radiations of *Drosophila* [68], the Amazon basin has the highest species diversity across the broadest taxonomic scope of any known region on this planet [3]. Just in the last 10 years, over 2000 new species have been described from the Amazon basin [69]. Reis et al. [5] report 4475 species described for the Neotropical region and estimate another 1550 undescribed species known from ichthyological collections.



(a)

(b)

Figure 2: Graphical representation of results of STRUCTURE analyses generated in the program DISTRUCT [58]. (a) represents individual level variation, while (b) represents population level variation.

Table 2: Analysis of molecular variance (AMOVA) of *Symphysodon* species, ESUs, phenotypes, and sampling localities. * = significant at $P = 0.05$.

Source of variation	SS	%
Among localities	711.61	29.43*
Within localities	1549.17	70.57*
Among species*	279.33	24.80*
Among localities within species	448.99	15.64*
Within localities	1578.28	59.56*
Among species†	374.83	21.67*
Among localities within species	353.49	13.69*
Within localities	1578.28	64.64*
Among phenotypes‡	416.82	19.54*
Among localities within phenotypes	311.50	13.48*
Within localities	1578.28	66.98*
Among phenotypes°	497.78	22.42*
Among localities within phenotypes	230.54	10.12*
Within localities	1578.28	67.47*
Among ESUs◊	484.96	22.41*
Among localities within ESUs	243.36	10.36*
Within localities	1578.28	67.27*

* = species *Symphysodon aequifasciatus* and *S. discus* [20].
† = species *Symphysodon tarzoo*, *S. aequifasciatus* and *S. discus* [17].
‡ = phenotypes Heckel, abacaxi, green, blue and brown [18, 54].
° = phenotypes Heckel, abacaxi, green, blue and brown, and Xingu [15].
◊ = ESUs of *Symphysodon* identified in the present study.

The Amazon basin also has a complex history that reflects a mix of Miocene geomorphological events and Plio-Pleistocene climatic oscillations [70]. All these events have left an impact on Amazonian ichthyofauna and the fauna and flora of the region, in general. The Amazon basin is also very large, encompassing over 6.87 million km^2. Probably no species has a basin-wide distribution, but there are a large number of broadly distributed fish species and species complexes [5]. This inevitably results in large census sizes, and in many cases also in large effective population sizes, for example, [71]. From a population genetic perspective, the time to speciation, that is, reciprocal monophyly, is directly proportional to effective population sizes, having a 95% probability of occurring within 2.2 *Ne* generations for mtDNA to upwards from 8.7 *Ne* generations for just one nDNA locus [72]. Assuming that many of the fish species of the Amazon basin have large effective population sizes, one is poised with an additional difficulty of recognizing species that are nonmonophyletic, and distinguishing these species from intraspecific geographic variants.

Haplotype sharing appears to be a relatively common phenomenon in Amazonian fishes. Examples include extensive interspecific haplotype sharing in the genera *Cichla* [10, 11], *Potamotrygon* [12], *Symphysodon* [15], *Serrasalmus* [13], and *Piaractus* [14]. Hybridization and incomplete lineage sorting have been invoked as explanations of the pattern of haplotype sharing, but irrespective of the ultimate cause, interspecific haplotype sharing makes recognition and delimitation of species and evolutionary significant units (ESUs) difficult. Monophyly is a convenient operational criterion for recognition of species [73] and ESUs [74], but monophyly is neither necessary nor sufficient for inference of species or ESUs. Broadly encompassing or primary species concepts such as the evolutionary species concept are difficult to apply due to lack of an all-encompassing operational

Table 3: Proportion of each biological group (PURPLE, GREEN, RED, and PINK—see Figure 2) in each of the five ESUs. Heterogeneity—heterogeneity in genetic composition.

	PURPLE	GREEN	RED	PINK	Heterogeneity
Heckel+abacaxi *Symphysodon discus*	0.886	0.025	0.073	0.016	0.309
Green *Symphysodon tarzoo*	0.011	0.948	0.029	0.013	0.104
Blue *Symphysodon* sp. 1	0.010	0.142	0.821	0.026	0.473
Brown *Symphysodon aequifasciatus*	0.030	0.087	0.836	0.047	0.409
Xingu *Symphysodon* sp. 2	0.009	0.064	0.055	0.872	0.369

criterion. However, incorporating both evolutionary as well as ecological information in inferring species and ESUs is crucial. To this end, Crandall et al. [61] proposed to test for recent as well as historical genetic and ecological exchangeability as a criterion for inferring cohesion sensu Templeton [75] between studied groups. The flexibility of this approach allows the identification of intraspecific structuring, of ESUs, of species experiencing different degrees of evolutionary isolation, as well as cases where formerly distinct species have recently lost their evolutionary distinctness.

Among the different ways to distinguish between recent and historical genetic exchangeability, Crandall et al. [61] propose to use mtDNA to test historical genetic exchangeability and microsatellite loci to test recent genetic exchangeability. We use this recommendation, since analyses of the mtDNA data were phylogenetic [15], and thus conveyed information about lineages and history of these lineages, while analyses of microsatellite data focused on current system of mating of the studied groups (this study). For recent exchangeability, we analyzed the microsatellite data collected in this study in the program STRUCTURE [55]. The number of biological groups observed in our study sample was inferred using the methodology of Evanno et al. [59] and we inferred four biological groups. The algorithm in STRUCTURE takes into account both Hardy-Weinberg equilibrium and linkage disequilibrium among loci (correlated allelic frequencies within biological groups); it therefore makes inferences about recent patterns of mating. The methodology of Evanno et al. [59] takes into account that most natural populations exist as metapopulations, that is, that species comprise partially differentiated groups, which themselves are composed of smaller but much more weakly if at all differentiated groups. In our analyses, we assumed that individuals could be admixed; that is, individuals' genetic composition could be the result of the contribution of more than one biological group. Phenotypic groups could then be composed of admixed individuals, but different phenotypic groups could have different patterns of admixture. Analyses of recent genetic exchangeability indicated the presence of four biological groups, two of which corresponded to recognized phenotypes (green and Heckel+abacaxi; the Heckel and the abacaxi groups belong to the same biological group), and one of which corresponded to the Xingú group (Xingú clade [15] and the Cametá locality). The fourth biological group formed the majority portion of genomes of individuals comprising the blue and brown phenotypes, but many individuals of both phenotypes were admixed with other biological groups. However, the patterns of admixture between the blue and the brown groups were different ($P = 0.0397$).

For historical exchangeability, we used the mitochondrial DNA results of Farias and Hrbek [15]. The phylogenetic results reported in that study reflect the evolutionary history of the *Symphysodon* species complex on the time scale of the coalescent, and therefore were used to infer historical exchangeability among the groups. Historically differentiated groups corresponded to the green, the blue and the Xingú groups, while the brown and the Heckel groups showed extensive haplotype sharing, but significant differences in allelic frequencies [15]. All abacaxi individuals had common brown haplotypes.

The mitochondrial and microsatellite DNA genetic patterns observed in the blue and brown phenotypes are not concordant. The blue phenotype forms a distinct mitochondrial clade supported by numerous molecular synapomorphies [15]. The blue and brown phenotypes also have subtle differences in color and color patterns, yet from the microsatellite nDNA perspective, they are only weakly divergent from each other as a result of different patterns of admixture of the RED biological group with other biological groups. However, in spite of potentially ongoing gene-flow between the blue and the brown phenotypes at the nuclear DNA level, this geneflow has not impacted the cohesiveness and evolutionary distinctness of the two phenotypes.

The mitochondrial and microsatellite DNA genetic patterns observed in the Heckel+abacaxi and the brown phenotypes also are not concordant. However, in this case, the Heckel+abacaxi and the brown phenotypes represent distinct biological groups, PURPLE and RED, respectively, but with extensive mtDNA haplotype sharing between the phenotypes [15] and the presence of admixed individuals.

Inference of potential recent ecological exchangeability was based on the types of water inhabited by different groups of *Symphysodon*. *Symphysodon* species live in lentic

Table 4: Indexes of genetic diversity at the 13 microsatellite loci used for the analysis of the 24 localities of *Symphysodon* phenotypes. A: number of alleles; H_O: observed heterozygosity; H_e: expected heterozygosity. P: probability that H_e and H_O are not different.

							Locus							
POP		Sd04	Sd05	Sd08	Sd10	Sd11	Sd12	Sd14	Sd15	Sd22	Sd23	Sd25	Sd27	Sd30	Total
	A	2	3	2	2	4	2	4	12	3	2	1	3	4	43
TB	H_O	0.800	0.133	0.933	0.067	0.467	0.600	0.333	0.933	0.400	0.133		0.400	0.533	0.491
15	H_e	0.515	0.131	0.515	0.067	0.467	0.508	0.402	0.903	0.441	0.129		0.432	0.559	0.450
	P	0.019	0.994	0.001	0.894	0.837	0.390	0.789	0.332	0.320	0.782		0.810	0.966	
	A	2	2	2	1	5	2	3	10	4	3	2	1	3	39
JT	H_O	0.800	0.200	0.900		0.600	0.400	0.300	0.800	0.400	0.100	0.100		0.500	0.418
10	H_e	0.526	0.189	0.521		0.558	0.442	0.279	0.911	0.500	0.100	0.100		0.484	0.402
	P	0.058	0.725	0.010		0.868	0.880	0.958	0.426	0.094	0.868	0.868		0.675	
	A	2	1	2	1	4	3	2	6	3	2	2	3	2	33
JR	H_O	0.667		1.000		0.500	0.500	0.333	0.833	0.667	0.333	0.333	0.333	0.500	0.454
6	H_e	0.545		0.545		0.561	0.591	0.303	0.818	0.545	0.303	0.303	0.318	0.530	0.461
	P	0.414		0.014		0.466	0.828	0.624	0.913	0.682	0.624	0.624	0.971	0.944	
	A	4	5	4	4	7	2	5	16	4	4	3	2	7	66
TF	H_O	0.522	0.360	0.917	0.080	0.560	0.520	0.440	0.680	0.417	0.320	0.200	0.480	0.360	0.480
23	H_e	0.545	0.323	0.570	0.079	0.574	0.458	0.372	0.920	0.357	0.290	0.187	0.444	0.442	0.465
	P	0.897	1.000	0.009	0.998	0.523	0.428	0.996	0.001	0.948	0.989	0.958	0.607	0.615	
	A	4	4	3	3	4	3	2	13	3	5	2	3	11	60
JP	H_O	0.579	0.211	0.947	0.105	0.368	0.526	0.421	0.789	0.368	0.263	0.105	0.211	0.579	0.454
19	H_e	0.599	0.201	0.536	0.104	0.371	0.421	0.341	0.883	0.317	0.248	0.102	0.351	0.569	0.444
	P	0.489	1.000	0.002	0.996	0.771	0.489	0.245	0.065	0.809	1.000	0.809	0.224	0.929	
	A	4	1	2	1	4	2	1	6	1	1	1	2	5	31
CO	H_O	0.500		0.250		0.750	0.250		1.000				1.000	1.000	0.432
4	H_e	0.643		0.250		0.750	0.250		0.929				0.571	0.893	0.409
	P	0.227		0.775		0.677	0.775		0.679				0.046	0.629	
	A	4	2	3	1	8	3	2	15	2	2	2	4	14	62
PU	H_O	0.647	0.188	0.471		0.813	0.111	0.111	0.944	0.167	0.077	0.056	0.722	0.722	0.437
18	H_e	0.635	0.175	0.551		0.843	0.110	0.108	0.922	0.157	0.077	0.056	0.640	0.852	0.470
	P	0.780	0.679	0.710		0.472	0.996	0.803	0.080	0.700	0.885	0.904	0.545	0.060	
	A	4	2	3	2	5	2	3	8	4	3	2	2	10	50
IRMM	H_O	0.429	0.143	0.667	0.143	0.429	0.143	0.429	0.857	0.429	0.286	0.143	0.429	1.000	0.450
7	H_e	0.495	0.143	0.591	0.143	0.670	0.143	0.385	0.912	0.571	0.275	0.143	0.363	0.934	0.522
	P	0.361	0.839	0.421	0.839	0.064	0.839	0.914	0.676	0.827	0.978	0.839	0.471	0.633	
	A	4	3	3	1	10	3	4	16	2	4	3	3	14	70
MN	H_O	0.684	0.400	0.263		0.900	0.100	0.167	0.895	0.211	0.200	0.105	0.211	0.833	0.423
20	H_e	0.593	0.337	0.240		0.888	0.099	0.162	0.933	0.193	0.191	0.104	0.383	0.903	0.458
	P	0.450	0.741	0.933		0.334	0.997	1.000	0.140	0.608	1.000	0.996	0.064	0.308	
	A	2	3	1	2	2	3	3	7	2	2	2	1	8	38
NA	H_O	0.500	0.333		0.250	0.000	0.250	0.417	0.833	0.182	0.100	0.083		0.750	0.312
12	H_e	0.464	0.301		0.228	0.429	0.236	0.359	0.848	0.173	0.100	0.083		0.848	0.406
	P	0.665	0.923		0.621	0.046	0.970	0.842	0.871	0.740	0.868	0.880		0.876	
	A	3	5	3	3	4	3	4	11	3	4	3	2	9	56
BB	H_O	0.174	0.565	0.083	0.087	0.500	0.250	0.250	0.958	0.250	0.136	0.083	0.125	0.625	0.341
24	H_e	0.240	0.565	0.082	0.086	0.563	0.228	0.233	0.873	0.230	0.132	0.228	0.120	0.730	0.375
	P	0.185	0.845	0.997	0.997	0.075	0.921	0.998	0.518	0.921	0.990	0.002	0.744	0.006	
	A	3	2	1	1	2	1	3	8	2	1	1	1	4	30
DM	H_O	0.400	0.400			0.000		0.400	1.000	0.200				1.000	0.291
5	H_e	0.378	0.356			0.533		0.378	0.956	0.200				0.733	0.356

Table 4: Continued.

		Locus													
POP		Sd04	Sd05	Sd08	Sd10	Sd11	Sd12	Sd14	Sd15	Sd22	Sd23	Sd25	Sd27	Sd30	Total
	P	0.958	0.576			0.083		0.958	0.628	0.804				0.544	
	A	4	2	5	1	3	3	4	12	2	2	2	4	8	52
NAR	H_O	0.300	0.375	0.455		0.400	0.364	0.545	0.909	0.100	0.250	0.091	0.200	0.600	0.484
11	H_e	0.595	0.325	0.775		0.600	0.537	0.593	0.931	0.100	0.500	0.091	0.537	0.821	0.534
	P	0.024	0.514	0.399		0.644	0.297	0.680	0.425	0.868	0.187	0.875	0.107	0.476	
	A	4	3	3	2	6	2	2	9	2	3	2	3	6	47
NO	H_O	0.900	0.250	0.100	0.111	0.667	0.100	0.400	0.900	0.333	0.375	0.100	0.400	0.700	0.434
10	H_e	0.668	0.433	0.416	0.111	0.680	0.395	0.337	0.884	0.294	0.492	0.100	0.647	0.758	0.599
	P	0.608	0.042	0.018	0.860	0.159	0.020	0.429	0.772	0.549	0.767	0.868	0.246	0.945	
	A	2	2	3	1	2	3	2	8	1	2	3	1	4	34
AX	H_O	0.750	0.400	1.000		0.200	0.400	0.200	1.000		0.000	0.400		0.500	0.405
5	H_e	0.536	0.356	0.644		0.200	0.378	0.200	0.956		0.356	0.378		0.750	0.464
	P	0.230	0.576	0.172		0.804	0.958	0.804	0.628		0.025	0.958		0.544	
	A	4	2	4	2	6	3	2	11	2	2	1	3	9	51
UA	H_O	0.600	0.200	0.400	0.100	0.800	0.300	0.600	0.900	0.200	0.200		0.400	0.800	0.482
10	H_e	0.600	0.189	0.489	0.100	0.858	0.279	0.442	0.942	0.189	0.189		0.568	0.879	0.545
	P	0.002	0.725	0.930	0.868	0.074	0.958	0.175	0.433	0.725	0.725		0.343	0.100	
	A	6	3	5	1	7	2	4	15	3	4	2	5	11	68
MA	H_O	0.611	0.111	0.529		0.706	0.278	0.389	1.000	0.278	0.278	0.056	0.667	0.778	0.486
18	H_e	0.743	0.110	0.631		0.795	0.322	0.459	0.927	0.252	0.257	0.056	0.756	0.890	0.552
	P	0.598	0.996	0.832		0.828	0.631	0.067	0.461	0.926	0.998	0.904	0.711	0.355	
	A	3	4	3	1	7	3	3	14	4	2	4	4	12	74
NH	H_O	0.278	0.444	0.652		0.500	0.652	0.391	0.875	0.318	0.364	0.227	0.238	0.958	0.441
17	H_e	0.427	0.611	0.592		0.884	0.590	0.531	0.924	0.289	0.312	0.215	0.443	0.879	0.610
	P	0.179	0.890	<0.001		0.201	0.918	0.586	0.077	0.992	0.780	0.999	0.022	0.564	
	A	4	3	2	1	4	4	5	11	2	4	2	2	10	54
TR	H_O	0.350	0.450	1.000		0.188	0.200	0.650	0.800	0.056	0.316	0.056	0.050	0.500	0.409
20	H_e	0.406	0.535	0.513		0.546	0.345	0.526	0.868	0.056	0.360	0.056	0.050	0.777	0.469
	P	<0.001	0.710	<0.001		<0.001	0.274	0.914	0.461	0.904	0.978	0.904	0.909	<0.001	
	A	4	3	2	1	7	3	4	12	2	2	3	5	10	58
TP	H_O	0.813	0.188	0.125		0.688	0.375	0.500	1.000	0.133	0.063	0.125	0.688	0.750	0.472
16	H_e	0.647	0.179	0.226		0.808	0.401	0.421	0.879	0.129	0.063	0.123	0.718	0.738	0.482
	P	0.492	0.982	0.086		0.772	0.940	0.939	0.995	0.782	0.897	0.995	0.917	0.882	
	A	5	2	4	1	7	2	3	9	3	2	1	5	10	54
AL	H_O	0.385	0.077	0.231		0.846	0.231	0.154	0.769	0.250	0.154		0.692	0.846	0.399
13	H_e	0.566	0.077	0.566		0.831	0.409	0.151	0.880	0.236	0.148		0.662	0.828	0.504
	P	0.776	0.885	0.122		0.889	0.136	0.993	0.165	0.970	0.764		0.247	0.034	
	A	2	4	3	2	2	4	4	12	2	4	2	2	8	51
XI	H_O	0.421	0.350	0.350	0.053	0.150	0.100	0.300	0.800	0.050	0.200	0.100	0.200	0.600	0.320
20	H_e	0.444	0.314	0.456	0.053	0.142	0.191	0.276	0.931	0.142	0.276	0.097	0.185	0.697	0.385
	P	0.911	0.989	0.608	0.906	0.717	0.003	0.996	0.502	0.004	0.211	0.814	0.619	0.970	
	A	3	2	1	1	3	2	2	7	3	4	1	3	3	35
JA	H_O	0.667	0.333			0.444	0.333	0.333	0.889	0.111	0.889		0.444	0.889	0.475
9	H_e	0.582	0.294			0.386	0.529	0.503	0.850	0.307	0.608		0.386	0.569	0.445
	P	0.606	0.549			0.865	0.317	0.370	0.691	0.029	0.451		0.865	0.124	
	A	4	2	3	1	4	3	3	9	2	2	2	4	6	45
CA	H_O	0.867	0.133	0.933		0.667	0.467	0.333	0.800	0.333	0.286	0.067	0.333	0.733	0.505
15	H_e	0.641	0.129	0.605		0.614	0.384	0.453	0.887	0.287	0.254	0.067	0.306	0.648	0.462
	P	0.438	0.782	0.008		0.857	0.708	0.627	0.185	0.439	0.533	0.894	0.996	0.969	

	Heckel	Abacaxi	Green	Blue	Brown	Xingú
Heckel		Case 8	Case 1	Case 1	Case 2	Case 1
Abacaxi	− | − + | −		Case 1	Case 1	Case 2	Case 1
Green	+ | + + | +	+ | + + | +		Case 1/7	Case 1	Case 1
Blue	+ | + + | +	+ | + + | +	+ | +/− + | +/−		Case 1/3	Case 1
Brown	+ | + − | +	+ | + − | +	+ | + + | +	+/− | + + | +		Case 1
Xingú	+ | + + | +	+ | + + | +	+ | + + | +	+ | + + | +	+ | + + | +	

FIGURE 3: Diagnosis of phenotypes of *Symphysodon* using the methodology and criteria of Crandall et al. [61]. Lower diagonal—tests of hypotheses of genetic (left column) and ecological (right column) exchangeability during recent (upper row) and historical (lower row) times: + = null hypothesis rejected; − = null hypothesis not rejected. Upper diagonal—Inference of ESU categories: Case 1 = long separated species; Case 2 = distinct species; Case 3 = distinct populations (recent admixture and loss of genetic distinctness); Cases 7 and 8 = single population.

TABLE 5: Analysis of differences in physiochemical properties of water in which different groups of *Symphysodon* occur. Data were taken from Tables 3 and 4 of Bleher et al. [16]. Data are in online supplement 1. The abacaxi and Xingu phenotypes were not included in analyses due to small sample sizes. Because of linear separation of the independent variables (pH and conductivity) in tests involving the Heckel phenotype, it was not possible to include both variables in the same model, and therefore, variables were analyzed separately. Linear separation also occurred in pH due to nonoverlapping pH values for the Heckel and blue phenotypes.

	Heckel *Symphysodon discus*	Green *Symphysodon tarzoo*	Blue *Symphysodon* sp. 1
Green *Symphysodon tarzoo*	pH: $P = 0.022$ Cond: $P = 0.158$		
Blue *Symphysodon* sp. 1	pH: $P < 0.001$ Cond: $P = 0.144$	pH: $P = 0.514$ Cond: $P = 0.549$ pH : Cond: $P = 0.546$ Wald test: $P = 0.770$	
Brown *Symphysodon aequifasciatus*	pH: $P = 0.019$ Cond: $P = 0.004$	pH: $P = 0.013$ Cond: $P = 0.059$ pH : Cond: $P = 0.059$ Wald test: $P = 0.038$	pH: $P = 0.005$ Cond: $P = 0.021$ pH:Cond: = 0.020 Wald test: = 0.019

habitats associated with major bodies of water. They inhabit all three major types of Amazonian waters [62], however, because of the lentic character of the waters inhabited, the white-water type has little suspended sediment. The chemical characteristics of the tree principal water types are very different [62], and also contain distinct fish faunas. Furthermore, the chemistry of white-water of the Amazon is different from that of the Solimões. The Amazon River is formed at the confluence of the black-water Negro River and the white-water Solimões where the Solimões contributes 49%, the Negro 14% and other Guyana and Brazilian Shield rivers the remaining 27% of the volume of the Amazon [76]. The confluence of the Solimões with the Negro, and the formation of the Amazon corresponds to the boundary between the blue, the Heckel and the brown *Symphysodon* groups, respectively, and there are significant differences in pH and conductivity of *Symphysodon* habitats occupied in these three rivers (Table 5). The Xingú clade together with the Cametá locality occur in the clear water type of the Brazilian Shield, and thus also are likely ecologically differentiated from other groups. The southern and northern tributaries of the Amazon are further differentiated by hydrological regimes, which potentially create a temporal reproductive barrier. The parapatrically distributed green and blue phenotypes occur in the lentic habitats of the Solimões that based on their pH and conductivity appear not to be different; however, it is likely there are other ecological differences separating the green and blue phenotypes. The Heckel and the abacaxi groups both inhabit lentic black-water habitats; however, they occur in Guyana and Brazilian Shield drainages, respectively. It is unclear whether these groups are ecologically exchangeable; however, it is worth noting that the geographic distribution of the abacaxi group is restricted to few affluents of the lower Madeira River. These same affluents contain ichtiofauna shared with the Negro River, for example, *Cichla temensis* [10], contain at least one species of *Rivulus* (TH pers. obs.) from the Guyana Shield clade of *Rivulus* [77], which may indicate historical connection of the lower Madeira River region with the Negro River basin.

No data exists on historical ecological exchangeability of the different *Symphysodon* groups, however, actual courses of Amazonian rivers and their headwaters in the three main geological formations of the Amazon basin, the Andes mountains, and the Guyana and Brazilian shields, have assumed their current forms at least six million years ago [78] although on a more regional scale, there has been much dynamism throughout the Pleistocene [79]. One can also argue from the principle of uniformitarianism and phylogenetic niche conservatism that current patterns of ecological association reflect historical patterns of ecological associations.

Analyses of recent and historical ecological and genetic exchangeability permits us to diagnose ESUs and infer intraspecific population structure [61]. A summary of the diagnosis of *Symphysodon* phenotypes is presented in Figure 3. Based on the criteria of Crandall et al. [61] all comparisons resulting in Case 1 differentiation should be considered distinct species. Case 1 [61] is observed between the green and all other phenotypes but blue, between the brown and all other phenotypes, between the Heckel and all other phenotypes, between the abacaxi and all other phenotypes but Heckel, and between the blue and all other phenotypes but brown. Differentiation was weak between the Heckel and abacaxi phenotypes (Case 8) suggesting that both phenotypes belong to the same species, but represent distinct populations. Whether the blue versus brown phenotype comparison represents Case 1 or Case 3 depends on how one quantifies recent genetic exchangeability. One can either consider this hypothesis not rejected (both phenotypes are predominantly biological cluster RED) or as rejected (patterns of genetic admixture are different). We opt for rejecting the null hypothesis of recent genetic exchangeability given that the mtDNA haplotypes of both phenogroups are geographically restricted and nonoverlapping, and therefore there is either no ongoing geneflow, there is no ongoing geneflow at adaptive loci, or selection removes the "wrong" mtDNA haplotype if geneflow occurs. Based on the proxy variables of pH and conductivity, there appear to be no barriers to ecological exchangeability between the green and blue phenotypes (Case 7); however, both groups maintain their evolutionary distinctness and are parapatrically distributed, suggesting that there likely are other ecological barriers not analyzed in this study (unobserved Case 1).

The results of the genetic and ecological exchangeability tests are summarized in Figure 3, and indicate the presence of five evolutionary species comprising the genus *Symphysodon*. The Heckel, Xingú, green, blue, and brown phenotypes represent full-fledged evolutionary species. The blue and the brown phenotypes probably are experiencing gene-flow but are maintaining their independent evolutionary trajectories, while the brown group has undergone historical admixture with the Heckel+abacaxi and the Xingú group. Despite being allopatric, the Heckel and the abacaxi phenotypes do not represent independent biological entities, possibly due to recent geographic separation.

The notion that the five phenotypes represent biological species rather than intraspecific variation is also supported by the amount of genetic divergence between the phenotypes. We observed interphenotype F_{ST} values between 0.02 and 0.38 (online supplement S3). All intraphenotype F_{IS} values averaged at 0.09. These F values are comparable to values reported in other studies of fish that used microsatellite markers to analyze recently diverged species groups. For example, Barluenga et al. [80, 81] observed in the Central American Midas cichlid complex (*Amphilophus citrinellus*, *A. labiatus*, and *A. zaliosus*) interspecific F_{ST} values ranging from 0.01 to 0.35, and intraspecific values no larger than 0.08. Similarly, an analysis of a *Sebastes* species complex (*S. fasciatus, S. mentella, S. marianus* and *S. viviparus*) resulted in interspecific pair-wise F_{ST} values ranging from 0.12 to 0.50 [82], while interpopulational pairwise F_{ST} values were never larger than 0.04 [82].

4.1. Taxonomy of the Genus Symphysodon. Our analyses and diagnoses using the criteria of Crandall et al. [61] indicate the genus *Symphysodon* is comprised of five ESUs. There are several described species, and several specific names available; however, throughout the taxonomic history of the genus, there has been substantial confusion. Therefore, we reevaluate existing classification.

The first described species, and the type species of the genus is *Symphysodon discus* Heckel, 1840. The type specimen was collected at Barra do Rio Negro, and it represents the phenotype Heckel. *Symphysodon discus* occurs in the Negro River basin, and the Trombetas River [21]; however, based on this study as well as previous analyses [15, 16], *S. discus* also occurs in the Nhamunda and Uatuma River basins. The Nhamunda and Uatuma Rivers are the two principal drainages geographically located between the Negro and Trombetas Rivers, and all these rivers drain the Guyana Shield. In 1981 Burgess described a sub-species from the Abacaxis River (*Symphysodon discus willischwartzi* Burgess, 1981). Analyses of the microsatellite data and diagnoses of genetic and ecological exchangeability also indicate that the phenotype abacaxi represents the same taxon as *Symphysodon discus* Heckel, 1840. This conclusion is also supported by Kullander [21]. The abacaxis phenotype is allopatric to the Heckel phenotype and parapatric with the brown phenotype. Its distinguishing characteristic is its yellowish-reddish background body color [83] which has led some authors to suppose that the abacaxi phenotype is a hybrid between *S. discus* and *S. aequifasciatus* [84]. From a nuclear DNA perspective, the abacaxi phenotype sampled from the type locality is nearly pure *S. discus* while some individuals of the abacaxi phenotype from the region of Novo Aripuaña show a signature of genomic admixture with the brown phenotype. In spite of instances of probable hybridization, the genomic composition of the abacaxi phenotype is no different than that of the Heckel phenotype. However, the mitochondrial genome of the abacaxi phenotype has been replaced by the mitochondrial genome of the brown phenotype, most likely via introgressive hybridization with the brown phenotype. In summary, the Heckel+abacaxi phenotype/ESU is *Symphysodon discus*.

The second species of *Symphysodon* was described over sixty years later as *Symphysodon discus* var. *aequifasciatus* Pellegrin, 1904. Three individuals were used in its description, two from Tefé and one from Santarém. Tefé is within the geographic distribution of the green phenotype, while Santarém is within the geographic distribution of the brown phenotype. The differences reported in the original description of Pellegrin were used by Schultz [18] as basis for elevating *Symphysodon discus* var. *aequifasciatus* to the species level (*Symphysodon aequifasciatus*).

In 1959/1960 Lyons described the subspecies *Symphysodon discus tarzoo* that differed from *S. aequifasciatus* by the presence of red spots on its fins and body. Its description was based on specimens from Letícia, Colombia, and its characteristics are those of the green phenotype. Short time later Schultz [18] revised the genus *Symphysodon*, rejecting the name "tarzoo" and describing three subspecies of *Symphysodon aequifasciatus*. Schultz [18] described the subspecies *S. aequifasciatus axelrodi* (brown phenotype) designating a type from Belém (eastern Amazon), the subspecies *S. aequifasciatus haraldi* (blue phenotype) designating a type from Benjamin Constant (western Amazon), and restricted the nominal subspecies *S. aequifasciatus aequifasciatus* to the green phenotype designating a type locality as Tefé (western Amazon). The geographic distribution of the blue phenotype is the central Amazon, however. For these and additional reasons, both Bleher [19] and Kullander [21] doubt that the type specimens of *S. aequifasciatus haraldi* were collected at Benjamin Constant by Axelrod and Schultz as reported in Schultz [18].

There is little recent controversy with respect to the taxonomy of the green phenotype. It forms a well supported mtDNA clade [15–17], and our microsatellite DNA data indicate that it represents a biological entity that is clearly differentiated from other phenotypes. The classification of the western Amazon green phenotype is contentious, however.

Following the revision of Schultz [18], most authors, for example, [20, 21, 85] did not recognize the subspecific classification of Schultz [18]. Ready et al. [17] based on mtDNA and morphometric evidence recognized the green phenotype as a species, revalidating the name *Symphysodon tarzoo* Lyons, 1959, and designating a neotype (INPA 25960). However, Bleher et al. [16] rejected the name *S. tarzoo* in favor of *Symphysodon aequifasciatus* Pellegrin, 1904. These differences in classification of the green taxon can be attributed to several sources.

First, the magazine in which Lyons' article was published is dated 1960 (*Tropicals*—Holiday Issue—1960, Vol. 4, no. 3) rather than 1959, and therefore, *Symphysodon discus tarzoo* Lyons, 1960 would be a junior synonym of *Symphysodon aequifasciatus aequifasciatus* Schultz, 1960 published in the June issue of the Tropical Fish Hobbyist. However, the publication of Lyons' article must have preceded that of Schultz since Schultz himself [18] cites the Lyons' article as "Holiday issue 1960" followed in parentheses by the date "November 28, 1959," and then goes on to reject Lyons' description on the grounds that it does not satisfy standards for species descriptions by the International Rules of Zoological Nomenclature, now the International Code of Zoological Nomeclature (ICZN). However, whether Lyons' description was sufficient to meet ICZN standards is subjective since Lyons does have a description which includes diagnostic characters, and does provide a photograph of the new subspecies even if not of the type specimen, and therefore Ready et al. [17] consider Lyons' description valid. Bleher et al. [16] agree with Schultz's [18] assessment, and also make a second argument for rejecting the name *Symphysodon tarzoo* Lyons, 1959 on the grounds that Lyons did not explicitly name the new species. It is true that the description is not explicit and scientifically rigorous, however, according to the regulations of the ICZN, only after 1999 do species descriptions have to be explicit and intentional. Therefore, even if Lyons did not explicitly state that the name *tarzoo* referred to a new sub-species, the ICZN rule requiring to do so did not yet exist in 1959.

Further Bleher et al. [16] argue that when Pellegrin [86] was describing *Symphysodon discus* var. *aequifasciatus*, he intended to associate this name with the green phenotype since two of the three specimens in the type series are from Tefé (green phenotype) and were described by Pellegin before the one specimen from Santarém (brown phenotype). Still further, the authors argue that because Schultz [18] restricted *S. aequifasciatus aequifasciatus* to the green phenotype selecting lake Tefé as the type locality, but not designating a lectotype and that since Schultz's revision the name *aequifasciatus* has always been associated with the green phenotype and never with the blue or brown phenotypes, the name *Symphysodon aequifasciatus* should be the scientific name used for the green phenotype.

However, taxonomic rules are clear with respect to homonimies (Article 23.1 of the ICZN). If Pellegrin in 1904 described the variety *aequifasciatus* based on two different phenotypes (green and brown) which are now recognized as two species, and posteriorly Lyons in 1959 described the subspecies *tarzoo* using individuals of only the green phenotype, Lyons became the the first reviser, albeit unintentionally. Therefore, we follow this precedent of the first reviser of this taxon and adopt the name *Symphysodon tarzoo* Lyons, 1959, following the classification proposed by Ready et al. [17], for the green phenotype/ESU identified in this study.

A third and fourth biological species identified in our analyses are the brown and blue phenotypes. Both the brown and blue phenotypes are not genetically pure. In the case of the brown phenotype, many of its individuals are admixed principally with Heckel phenotype (*Symphysodon discus*) and the Xingú group phenotype, while in the case of the blue phenotype one observes admixture with the green phenotype (*Symphysodon tarzoo*). The blue phenotype forms a distinct mtDNA clade [15], while there is some haplotype sharing between the brown and Heckel phenotypes [15]. Bleher et al. [16] also observed that individuals of the blue/brown phenotype, all from the eastern Amazon, past the confluence of the Negro and Solimões Rivers, shared haplotypes with the

Heckel phenotype. The authors considered these individuals to be old hybrids, or more correctly be blue/brown individuals with introgressed Heckel mtDNA. The study of Ready et al. [17] sheds no information on the species status of the blue phenotype since individuals of this phenotype were not included in their analysis.

The classification of the brown and blue phenotype is also apparently controversial. This controversy stems directly from the controversy surrounding the classification of the green phenotype expounded on previously, the fact that Ready et al. [17] did not include the blue phenotype in their analysis, and that Bleher et al. [16] considered the brown phenotype to be the blue phenotype introgressed with mtDNA of the Heckel phenotype. Ready et al. [17] propose the name *Symphysodon aequifasciatus* for the brown phenotype and designate the Santarém individual from Pellegrin's type series (MNHN 1902-130) as lectotype, restricting the species *Symphysodon aequifasciatus* to the brown phenotype. However, since Bleher et al. [16] adopted the name *Symphysodon aequifasciatus* for the green phenotype, they then adopted the name *Symphysodon haraldi* for the blue and brown phenotypes. Although the type locality of *Symphysodon aequifasciatus haraldi* Schultz, 1960 (USNM 00179829) was reported as Benjamin Constant, this is highly doubtful based on several lines of evidence [19, 21] and material discussed in Bleher and Géry [87], leading Bleher and Géry [87] to propose Lake Beruri of the lower Purus River system as the correct type locality for Schultz's type. If Schultz's type (USNM 00179829) really originated from Lake Beruri in the lower Purus River system, the revisions of Ready et al. [17] and Bleher et al. [16] can be viewed as largely noncontradictory; however, ultimately, the true type locality of Schultz's type is unknowable at this point in time.

The results of our study indicate that while both the brown and blue phenotypes are derived from the same biological group, as phenotypic groups they show different patterns of admixture. The brown and blue phenotypes are also clearly differentiated at the mtDNA level [15]. Therefore *Symphysodon aequifasciatus* Pellegrin, 1904 (*apud*. Ready et al. [17]) should be restricted to the brown phenotype/ESU occurring in the eastern Amazon, downstream of the confluence of the Solimões and Negro Rivers, while the blue phenotype/ESU occurring in the central Amazon upstream of the confluence of the Solimões and Negro Rivers and east of the Purus Arch likely represents a scientifically yet to be described species of *Symphysodon*.

We also identified a fifth ESU in our analysis. This ESU is comprised of individuals from the Vitoria do Xingú (Xingú River) and the Cametá (Tocantins River) localities, both situated at the northern margins of the Brazilian Shield. The presence of this evolutionary entity was already observed by Farias and Hrbek [15], but neither the study of Ready et al. [17] nor that of Bleher et al. [16] include fishes from the Xingú or Tocantins drainages, and therefore neither study observed the presence of this group. No potential scientific name seems to exist for this taxon. Schultz in 1960 described the subspecies *Symphysodon aequifasciatus axelrodi* from Belém; however, Belém is a city on the southern Amazon River delta from which fish were exported, and *Symphysodon* species do not occur in the vicinity of the city. Geographically the closest region where the genus *Symphysodon* occurs is in the lower Tocantins River represented by the Cametá locality in our study, and drainages in the Portel region west of Belém. According to Bleher [19] the type specimen of *Symphysodon aequifasciatus axelrodi* Schultz, 1960 (USNM 00179831) looks most like fishes from Breves, Marajó Island (another region from which fish are exported but not collected), while Bleher and Géry [87]—within the book of Bleher [19]—postulate that the most likely region from where the type was collected is the lower Tapajós River where the brown phenotype occurs. If the type locality is the lower Tapajós River this would make *S. aequifasciatus axelrodi* a junior synonym of *Symphysodon aequifasciatus* Pellegrin, 1904 (*apud*. Ready et al. [17]). Rest of the type series is listed as having been collected from the lower Urubu River where the brown phenotype occurs. Again, the true location of the type locality is unknowable at this point in time, but ultimately has no bearing on the taxonomic status of the Xingú phenotype/ESU.

In summary, we conclude that the genus *Symphysodon* is comprised of five ESUs.

(i) *Symphysodon discus* Heckel, 1840 (Heckel and abacaxi phenotypes—western Guyana Shield—Negro and upper Uatuma, Nhamunda and Tombetas Rivers; western Brazilian Shield—Abacaxis River and some other blackwater affluents of the Madeira River).

Synonym: *Symphysodon discus willischwartzi* Burgess, 1981.

(ii) *Symphysodon tarzoo* Lyons, 1959 (green phenotype—western Amazônia—river drainages west of the Purus Arch).

Synonym: *Symphysodon discus* var. *aequifasciatus* Pellegrin, 1904 in part; *Symphysodon* Discus Tarzoo Lyons, 1959; *Symphysodon aequifasciatus aequifasciatus* Schultz, 1960 *Symphysodon aequifasciatus haraldi* Schultz, 1960? (in the unlikely assumption that Schultz's account of the type locality—Benjamin Constant—is correct); *Symphysodon aequifasciatus* in Bleher et al. [16].

(iii) *Symphysodon aequifasciatus* Pellegrin, 1904 (brown phenotype—eastern Amazônia—lower reaches of Amazon River and affluents east of the confluence of the Negro and Solimões Rivers).

Synonym: *Symphysodon discus* var. *aequifasciatus* Pellegrin, 1904 in part; *Symphysodon aequifasciatus axelrodi* Schultz, 1960? (if actual type locality is lower Tapajós River [87]); *Symphysodon haraldi* in Bleher et al. [16] in part.

(iv) *Symphysodon* sp. 1 (blue phenotype—central Amazônia—river drainage systems east of the Purus Arch and west of the Negro and Solimões River con fluence).

Synonym: *Symphysodon haraldi* in Bleher et al. [16] in part; *Symphysodon aequifasciatus* in Ready et al. [17] in part.

(v) *Symphysodon* sp. 2 (the Xingú group—eastern Brazilian Shield—lower Tocantins and Xingú Rivers).

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Review Article

The Monogenean Parasite Fauna of Cichlids: A Potential Tool for Host Biogeography

Antoine Pariselle,[1] Walter A. Boeger,[2] Jos Snoeks,[3,4] Charles F. Bilong Bilong,[5] Serge Morand,[1] and Maarten P. M. Vanhove[3,4]

[1] *ISE-M, UMR5554 CNRS, UR226 IRD (ex-ORSTOM), Université Montpellier II—CC 063, 34095 Montpellier Cedex 5, France*
[2] *Laboratório de Ecologia Molecular e Parasitologia Evolutiva, Grupo Integrado de Aquicultura e Estudos Ambientais, Universidade Federal do Paraná, Setor de Ciências Biológicas, Departamento de Zoologia, Caixa Postal 19073, CEP 81531-980, Curitiba, PR, Brazil*
[3] *Ichthyology Unit, African Zoology Department, Royal Museum for Central Africa, Leuvensesteenweg 13, 3080 Tervuren, Belgium*
[4] *Laboratory of Animal Diversity and Systematics, Biology Department, Katholieke Universiteit Leuven, Charles Deberiotstraat 32, 3000 Leuven, Belgium*
[5] *Laboratoire de Parasitologie et d'Ecologie, Département de Biologie et Physiologie Animales, Université de Yaoundé I, BP 812, Yaoundé, Cameroon*

Correspondence should be addressed to Maarten P. M. Vanhove, maarten.vanhove@bio.kuleuven.be

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We discuss geographical distribution and phylogeny of Dactylogyridea (Monogenea) parasitizing Cichlidae to elucidate their hosts' history. Although mesoparasitic Monogenea (*Enterogyrus* spp.) show typical vicariant distribution, ectoparasitic representatives from different continents are not considered sister taxa, hence their distribution cannot result from vicariance alone. Because of the close host-parasite relationship, this might indicate that present-day cichlid distribution may also reflect dispersal through coastal or brackish waters. Loss of ectoparasites during transoceanic migration, followed by lateral transfer from other fish families might explain extant host-parasite associations. Because of its mesoparasitic nature, hence not subject to salinity variations of the host's environment, *Enterogyrus* could have survived marine migrations, intolerable for ectoparasites. Host-switches and salinity transitions may be invoked to explain the pattern revealed by a preliminary morphological phylogeny of monogenean genera from Cichlidae and other selected Monogenea genera, rendering the parasite distribution explicable under both vicariance and dispersal. Testable hypotheses are put forward in this parasitological approach to cichlid biogeography. Along with more comprehensive in-depth morphological phylogeny, comparison with molecular data, clarifying dactylogyridean evolution on different continents and from various fish families, and providing temporal information on host-parasite history, are needed to discriminate between the possible scenarios.

1. Introduction: Explanations to the Current Distribution Pattern of Freshwater Fish Groups

Organisms with limited dispersal abilities are generally considered to be useful tools in historical biogeography. Examples include amphibians [1] and freshwater fishes [2, 3]. At the heart of many discussions on the evolutionary history and distribution patterns of major freshwater fish groups is the vicariance *versus* dispersal debate (e.g., [4, 5]). Although vicariance-based scenarios have classically been favoured, de Queiroz [6] gives an overview of how the importance of (often seemingly unlikely) dispersal events has been underestimated in historical biogeography, though his examples stem mostly from plants and terrestrial biota.

It is generally accepted that the distribution of several ancient freshwater groups such as Dipnoi (lungfishes) and Osteoglossiformes (bony tongues) results from major vicariant events after the breakup of Gondwana [7]. However, because of conflicting evidence, the discussion continues for more recent groups. Hertwig [8] discussed the biogeographic implications of the phylogeny of the Cyprinodontiformes (rivulines, killifishes, and live bearers). He explained their distribution by vicariance events in the basal clades, combined with subsequent dispersal, and stressed the discrepancy of the vicariance hypothesis with the paleontological record. For example, in aplocheiloid killifishes, the fossil record is much younger than the supposedly African-South American drift-vicariance event [9].

The same is true within the Characiformes (characins), for example, in Alestidae; Zanata and Vari [10] forwarded a clear vicariance hypothesis to explain their distribution and relationships. In addition, they mention typically vicariant biogeographic patterns in two other groups of Characiformes with African-South American sistergroup relationships. However, Calcagnotto et al. [11], in a molecular analysis, mostly confirming earlier morphology-based trees, concluded that marine dispersal cannot be excluded *a priori* and that a simple model of vicariance could not explain the biogeographic history of the order.

Within the Siluriformes (catfishes), the situation is more complex since several taxa are marine inhabitants. Sullivan et al. [12] could not confirm the existence of a supposedly trans-Atlantic clade suggested by others, but conversely, their overall tree of Siluriformes did not contradict a general vicariant distribution pattern of tropical freshwater catfishes either. A few recent publications have offered new angles to the vicariance *versus* dispersal debate. To explain the unexpected discovery of a Mesoamerican catfish within an African clade, Lundberg et al. [13] postulated a northern ancient intercontinental passage in warmer periods, including dispersal through freshened warm surface waters of the Arctic and adjacent oceans. Such episodic fresh surface waters have been suggested by Brinkhuis et al. [14] as an explanation for the presence of the freshwater fern *Azolla* and freshwater microfossils in Eocene marine deposits.

2. The Case of Cichlidae

For Cichlidae, the discussion is far from over either, as they exhibit a comparable biogeographic pattern found in Cyprinodontiformes, with basal lineages occurring in India and Madagascar, and the common problem of discordance between their fossil record and their age under the vicariance hypothesis [8]. Most recent arguments, however, seem to favour the vicariance model (e.g., [15–17]), but the dispersal model could not be eliminated. In fact, the numerous studies that have examined the fossil record and morphological and/or molecular phylogenies of the world's cichlid fauna (e.g., [9, 15, 17–29]) have not been able to end all doubts on the choice between the two main hypotheses explaining their current distribution pattern.

The first hypothesis postulates that the cichlid fishes originated ca. 130 million years ago (MYA) in Gondwana. Their current disjunctive distribution area (Africa, including Madagascar; South and Central America, Texas and the Caribbean; southern mainland India and Sri Lanka; the Levant and Iran [30]) comprises mostly Gondwanan regions; hence cichlids would have already been present in the major part of their current range before the splitting up of this super-continent. As for their non-Gondwanan occurrence in Texas, the Caribbean, the Levant, and Iran, migration through river systems over more recent landbridges is assumed (e.g., [16]) though the dispersal mode is still debated for Central America and the Caribbean [31, 32]. This implies that cichlid evolutionary history and present large-scale distribution on the continents resulted from major vicariant events and that cichlids did not need to cross extensive marine barriers to reach these distribution areas.

The second hypothesis, in line with the fossil data, suggests that cichlid fishes originated near Madagascar, or more precisely, that the cichlids living there belong to ancestral lineages, in view of their paraphyly ([25, 33, 34] and references therein). Consequently, cichlids would have secondarily colonized their current disjunctive distribution areas [24–26]. This scenario assumes that cichlid evolutionary history and present distribution have resulted from dispersal across various marine water channels.

According to Chakrabarty [15], the only way to resolve the controversy of the origin of Cichlidae (except for the discovery of a cichlid fossil older than 65 MY) is to falsify either of the two hypotheses. As the dispersal hypothesis is untestable because any distribution pattern can be explained by dispersal, Chakrabarty [15] suggested four potential means to show the vicariance hypothesis incorrect. Three of these falsifiers are based on the demonstration of an incompatibility between the timing of two supposedly concomitant events (one linked to the divergence of lineages, the other to geological processes); the fourth one includes the discovery of the same cichlid species on both sides of a supposed barrier to dispersal.

One of the potential "timing" falsifiers involves the use of molecular clock estimations, the absolute accuracy of which still remains unknown (e.g., [35] *versus* [36, 37]). However, Azuma et al. [17] made a strong case in calculating divergence times of major cichlid lineages, based on molecular evolutionary rates of large mitogenomic datasets of six cichlid species, leading to additional support for the vicariance hypothesis. As for the discovery of a new cichlid species on both sides of a marine channel, the likelihood is very low. The same applies to the finding of a cretaceous cichlid fossil. In comparison with other freshwater fishes spanning such a period of time, this would indeed imply a relatively large gap in the fossil record [25]. Furthermore, the advanced position of perciformes in teleost phylogeny (several higher level taxa encompassing the cichlids do not appear before the Gondwanan breakup) and the extensive fossil record available of several cichlid lineages make such a discovery improbable [38].

3. Parasites as an Additional Source of Information

Another solution might come from the use of a separate and independent data set related to cichlids. It is well established that parasites can furnish information on their hosts' ecology and (past and current) distribution [39–42]. Following Hoberg [43] and Nieberding and Olivieri [42], we could use parasites as keystones in biogeography, or proxies, to provide a new dimension to understand ecological interactions, distribution patterns, and the history of geographic regions and biota.

Some work has been done in this framework to infer host biogeography [44–55]. More specifically, interesting examples include the reconstruction of biogeographical events through the analysis of parasite communities [56–59] and the detection of patterns at a higher resolution than host genetics would permit [60–62]. Parasite phylogenetic data can also yield supporting or complementary information on host phylogenies [63–67]. Surprisingly, few studies refer to fishes and their associated monogenean parasites whereas these organisms seem useful as indicators of host biogeography and phylogeny. Indeed, they are strictly parasitic (i.e., adults cannot survive for a long time as free-living organisms), holoxenous (i.e., they have a direct lifecycle, with a short free-living larval stage which actively infests a new host), and generally oioxenous (i.e., parasite species are often species specific with regard to their host). A limited number of cases were made using these parasites, applying them in the fields of genetic population substructuring [68], historical patterns of their hosts' dispersal [69] or distribution [70], (co-)phylogenetic patterns [71–73], and host identification [74].

Pariselle et al. [75] used data from West African cichlids and concluded that monogenean species can behave similarly to the alleles of genes of their fish hosts. Distribution, and therefore biogeography, of the hosts will directly affect that of their monogeneans. Conversely, information provided by these parasites should be very useful for studies in fish biogeography. Importantly, as demonstrated by Pérez-Ponce de Léon and Choudhury [59] for South and Central American cichlids, a phylogenetic study of both host and parasite taxa is paramount to infer hypotheses on historical biogeography. Evidently, a geographically restricted availability of parasite records can seriously hamper an analysis. For example, theories on the "original" host, needed to infer host-switching or dispersal pathways, are hard to infer when certain regions or host taxa are undersampled. In addition, insufficient sampling ensues obvious problems related to how "real" absence data are when considering community composition [58].

Here we illustrate how parasite information may complement the discussion on vicariance *versus* dispersal hypotheses for cichlid age and distribution processes.

4. The Monogenean Parasite Fauna of Cichlids

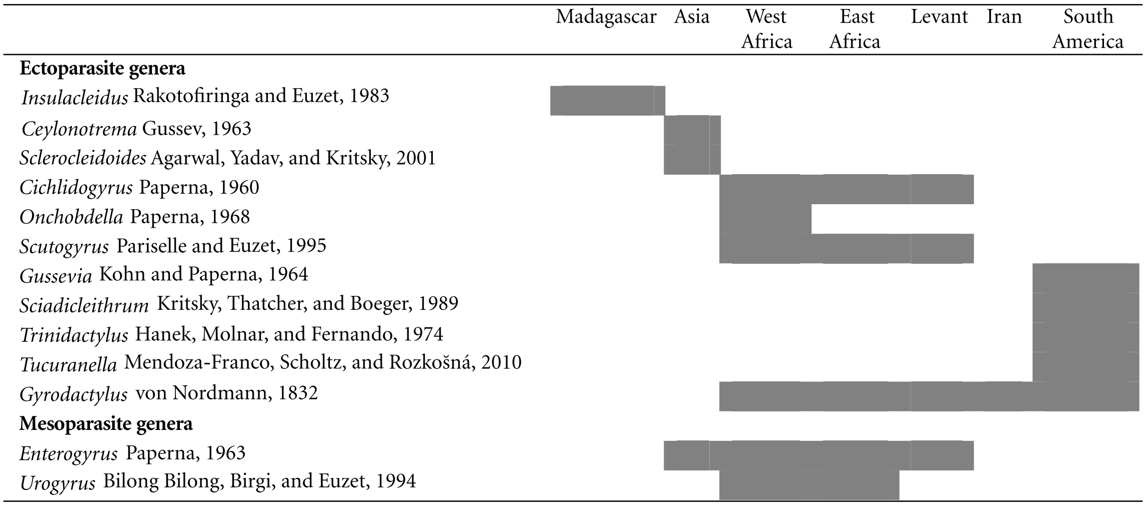
4.1. Data Collection on Cichlid Parasites. Because of the oioxenous host specificity of many monogenean species, the (geological) time scale and the biogeographical distribution of their hosts (no genus is represented naturally on two different continents) [76, 77], the approach used within the framework of this study is based on the generic rather than the species level. Some ancyrocephalid monogeneans from South American and African cichlids are known to exhibit a relatively low host specificity, infesting several host species [78, 79]. However, their otherwise often narrow species specificity would, in our view, render examining the parasite data at species level equivalent to the use of autapomorphic characters (instead of synapomorphic ones) in a phylogenetic tree, which is not appropriate [73] to infer the biogeographical history of the host. Furthermore, there are considerable differences in the number of parasite species reported from the various host genera. In view of the relatively low proportion of cichlid species worldwide examined for the presence of Monogenea, this imbalance is more likely to reflect differential sampling effort than variations in species richness between genera. Conversely, on the genus level, we feel confident the current state-of-the-art approaches the actual parasite diversity on cichlids to a higher extent.

To the best of our knowledge, Figure 1 provides a complete overview of the 13 monogenean genera infecting cichlids. Unfortunately, a considerable proportion of available data is fragmentary and based on studies on a limited number of regions or host species. The most exhaustively studied assemblage is probably Ancyrocephalidae from West African tilapias. The exemplary nature of monogenean records worldwide is demonstrated by the fact that, while there are revisions and phylogenies available for certain well-defined taxa (e.g., Diplectanidae [80], Capsalidae [81]), comprehensive worldwide information on Monogenea of an entire fish family is extremely rare.

The worldwide distribution of monogenean genera described from cichlid hosts shows a clear difference between the distributions of ecto- (i.e., living on the host, directly in contact with the environment) and mesoparasite (i.e., living in a host body cavity and not in direct contact with the external environment [91]) genera. The mesoparasite *Enterogyrus* is present in Asia, the whole of Africa, and the Levant. Pariselle and Euzet [78] hypothesize that the Asian and African-Levantine representatives of this genus might be considered as belonging to separate genera, on the basis of the possession of two *versus* one haptoral transversal bar. However, the overall similarities in haptor and even male copulatory organ structure suggest the various *Enterogyrus* spp. to be very closely related anyway.

In contrast, all ectoparasitic genera are endemic to the continent where they occur. Two genera currently seem to display a more restricted distribution: *Onchobdella* and *Urogyrus*. The first one was expected, like other African ectoparasitic genera, to be present on the entire continent, and not only in West Africa, but was found to be specific to hosts whose distribution is limited to this region (*Hemichromis* Peters, 1858 and *Pelmatochromis* Steindachner, 1894). *Urogyrus*, being a mesoparasite like *Enterogyrus*, could potentially be present on several continents, but is only found on hosts whose distribution is restricted to Africa (including haplochromines from the East African Great Lakes [92]).

While the above-mentioned cichlid parasites are mostly dactylogyridean Monogenea, the nominal (albeit probably paraphyletic [93]) genus *Gyrodactylus* is a member of the Gyrodactylidae, first proposed by Van Beneden and Hesse



	Madagascar	Asia	West Africa	East Africa	Levant	Iran	South America
Ectoparasite genera							
Insulacleidus Rakotofiringa and Euzet, 1983	▨						
Ceylonotrema Gussev, 1963		▨					
Sclerocleidoides Agarwal, Yadav, and Kritsky, 2001		▨					
Cichlidogyrus Paperna, 1960			▨	▨	▨		
Onchobdella Paperna, 1968			▨				
Scutogyrus Pariselle and Euzet, 1995			▨	▨	▨		
Gussevia Kohn and Paperna, 1964							▨
Sciadicleithrum Kritsky, Thatcher, and Boeger, 1989							▨
Trinidactylus Hanek, Molnar, and Fernando, 1974							▨
Tucuranella Mendoza-Franco, Scholtz, and Rozkošná, 2010							▨
Gyrodactylus von Nordmann, 1832			▨	▨	▨	▨	▨
Mesoparasite genera							
Enterogyrus Paperna, 1963		▨	▨	▨	▨		
Urogyrus Bilong Bilong, Birgi, and Euzet, 1994			▨	▨			

Figure 1: Current state of knowledge on biogeographical distribution of monogenean genera from cichlid fishes (fields shaded in grey indicate presence), compiled on the basis of [78, 82–90] and additional unpublished data from the authors. Note that sufficient data are clearly lacking for some regions, for example, on whether or not Malagasy and South American cichlids host mesoparasitic Monogenea. Recent synonymisations are taken into account, as are *nomina inquirenda*, for instance *Oreochromogyrus* Ferdousi and Chandra, 2002, which most likely concerns misidentified *Cichlidogyrus* larvae [78]. Only records from hosts occurring under natural conditions on the respective continents are included.

[94]. Moreover, despite its extensive morphological plasticity (*cfr. infra*), *Gyrodactylus* species are rather conservative on the scale of the characters used in this study. Given the limited number of representatives known from cichlids, this genus would not yield a high resolution in a morphological phylogeny. As we need a cladistic analysis of the genera under study, *Gyrodactylus* will not be included in our investigation. In contrast, it should be noted that *Gyrodactylus* in itself could be a useful biogeographic tool [95], as can other gyrodactylids [70]. However, the limited molecular data available on African and South American *Gyrodactylus* species do not show any close affinities between them either [82].

4.2. Phylogenetic Analysis. A preliminary phylogenetic analysis was performed using selected morphological characters available in the literature. Genera included are all those known to parasitize cichlids and selected parasites from marine and freshwater perciform fishes (Table 1) from the different continents home to cichlids. *Quadriacanthus*, infesting siluriformes, was added as its representatives depict similar and comparable morphological features (e.g., hook morphology). The inclusion of these taxa allowed a preliminary test of the monophyly of the parasites of Cichlidae, which would be the expected pattern under cospeciation (either by vicariance or dispersal). The addition of genera from other fish groups provided a preliminary test that the parasitic fauna of cichlids could encompass sister lineages to parasites of other sympatric fish species, especially if the hypothesis of parasite loss during dispersal in marine waters was supported.

The hypothesis on their relationship was proposed based on parsimony analysis of 17 unordered homologous series (Appendix). The chosen putative homologous series are those considered less prone to errors introduced by incomplete or questionable interpretations of morphology in the original descriptions of the species. Five of the homologous series used pertain to the copulatory complex; all others,

Table 1: Genera from non-cichlid hosts included in the phylogenetic analysis, with their host range.

Genus	Host fish families	Reference
Diplectanum Diesing, 1858	Muraenesocidae, Gerreidae, Kuhliidae, Latidae, Lutjanidae, Moronidae, Percichthyidae, Polynemidae, Priacanthidae, Sciaenidae, Serranidae, Sillaginidae, Sphyraenidae, Synancejidae, Terapontidae, Toxotidae, Cynoglossidae, Bagridae	[96]
Mastacembelocleidus Kritsky, Pandey, Agrawal, and Abdullah, 2004	Mastacembelidae	[97]
Euryhaliotrema Kritsky and Boeger, 2002	Sciaenidae, Haemulidae, Sparidae, Lutjanidae	[69]
Chandacleidus Agrawal, Tripathi, and Devak, 2006	Freshwater Ambassidae	[98]
Duplaccessorius Viozzi and Brugni, 2004	Freshwater Percichthyidae	[99]
Protogyrodactylus Johnston and Tiegs, 1922	Marine Terapontidae; Marine and brackish water Gerreidae	[100, 101]
Quadriacanthus Paperna, 1961	Clariidae and Bagridae	[102]

to the haptoral elements. Although both haptoral and copulatory complex, next to soft body parts, are important in Monogenea systematics [103], many other scientists focus their analyses solely on the attachment organ (e.g., [104–106]). Its complexity and variability, as well as the higher number of comparable elements, make it easier to extract coded characters from the haptor than from the genitals. Moreover, several studies comparing morphological and molecular data in Ancyrocephalidae suggest that the attachment organ structure mirrors phylogenetic relationships on the level between genera or major lineages while the copulatory organ is more suitable for distinguishing closely related species [107, 108]. The phylogenetic hypothesis was constructed in PAUP* v.4.0 b10 [109], with the Bremer support index [110–112] calculated with the help of TreeRot v.3.0 [113]. Initial analyses, using heuristic search (under a tree-bisection and reconnection branch swapping algorithm with 1000 random-addition-sequence replicates) provided individual consistency indices for character states used in successive weighting procedures [114] until values of the overall consistency (CI: [115]) and retention indices (RI: [116]) stabilized.

A total of 21 equally parsimonious trees (EPT) resulted from the analysis of parsimony (length = 13.97; CI = 0.81; CI excluding uninformative characters = 0.76; RI = 0.85). The strict consensus cladogram, summarising the most parsimonious phylogenetic relationships recovered from all EPT between the analysed genera is presented in Figure 2. The genera with species limited to Cichlidae (shaded genera names on Figure 2) are represented by up to three independent groups. The phylogenetic hypothesis does not support a single monophyletic assemblage of parasites of Cichlidae.

5. Information to Be Drawn from Cichlid Monogeneans

Our review shows that dactylogyridean ecto- and mesoparasites of cichlid hosts differ in their distributions. This raises the question as to which of the above-mentioned hypotheses on the biogeographic pattern of cichlids best explains this distributional incongruence. We would expect similar patterns in their geographic distribution, assuming that all lineages, meso- and ectoparasitic, have an equally long association with their cichlid hosts, a similar evolutionary rate, and have equally been subjected to the same biogeographical and coevolutionary processes/events. The African ectoparasitic *Cichlidogyrus* (as well as the mesoparasitic *Enterogyrus*) was found to infect South American cichlids following introduction of their African hosts, and the American *Sciadicleithrum* was reported from African cichlids under artificial conditions [117]. This supports the view that the evolutionary divergence between African and Neotropical cichlids is not the reason they do not share ectoparasites.

Thus, it is likely that the source of discrepancies between the distribution patterns observed for meso- and ectoparasite s resides in the fact that environmental factors may influence these parasite communities differently (e.g., [118]). In the framework of the dispersal hypothesis (see [25]), there is a single such factor that may influence the two types of parasites in two different ways. This theory assumes that there were successive migrations, taking place in different environmental conditions. Some hosts are assumed to have crossed marine waters (between Madagascar and Asia; Madagascar and Africa; West Africa and South America; Figure 3, black arrows) while others only used freshwater dispersal pathways (dispersal within Africa or South America; Figure 3, white arrows). Ectoparasites are directly affected by changes in the environment while mesoparasites such as *Enterogyrus* found in the stomach are not. Does this differential exposure to saline water allow us to favour one of both hypotheses on cichlid history? A couple of issues need to be addressed before this question can be answered.

5.1. Salinity Tolerance of Cichlidae and Monogenea. Many cichlids exhibit tolerance to a broad salinity range and some even display a mostly brackish lifestyle, such as representatives of the Asian *Etroplus* Cuvier, 1830, the only Iranian endemic cichlid *Iranocichla hormuzensis* Coad, 1982, and some African, Malagasy, and Neotropical species ([25] and references therein, [30, 32, 119–129]). Murray [25] and Briggs [9] used this ability as an important argument in favour of the recent dispersal hypothesis, but Sparks and Smith [16] contradicted the long-time survival of any cichlid species in saltwater conditions. Although, at least under natural conditions, there are no fully marine cichlid species at present, several wild populations occur in (highly) saline environments (e.g., *Sarotherodon melanotheron* Rüppell, 1852 in the Gambia and Senegal rivers [130], in Hann bay (off Dakar), Senegal and in Saint Jean bay, Mauritania; *Tilapia guineensis* (Günther, 1862) in Hann bay (off Dakar), Senegal (A. Pariselle, pers. obs.)).

Cichlids were possibly able to survive in marine environments, but this may not have been the case for their monogenean parasites, whose tolerance to salinity variations is generally shown to be low. Indeed, osmotic shocks are commonly used as a treatment against ectoparasitic Monogenea [123, 131–134]. Pariselle and Diamanka [135] showed that *S. melanotheron* lost all monogenean gill parasites with the increase of water salinity (>35 g/L), both in a natural environment and under experimental conditions.

On the other hand, *Enterogyrus* has been reported from highly saline waters [83]. The biogeography of mesoparasites (*Enterogyrus*) seems to display a vicariant history, as the same genus is present in Asia and Africa (Figure 1). The lack of shared ectoparasites between continents and the most often limited (as compared to their hosts') salinity tolerance of *Cichlidogyrus* is mentioned by Paperna [83] as an argument for the marine dispersal theory. Indeed, one could hypothesize that marine dispersal events in cichlids caused them to lose their ectoparasites, while retaining their mesoparasites, unexposed to the saline water. This could explain the incongruence between the distribution patterns of ecto- and mesoparasitic Dactylogyridea. However, for this conclusion to be drawn, it is necessary to know the interrelationships between the various monogenean genera under study.

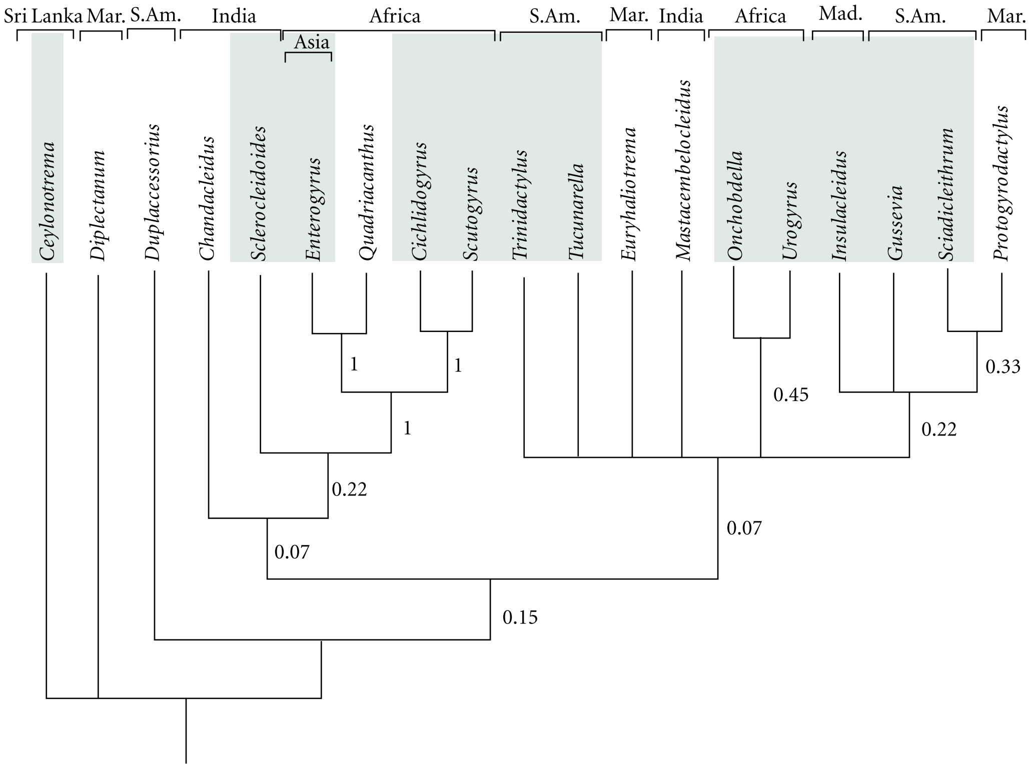


FIGURE 2: Strict consensus cladogram (of 21 EPT) depicting the putative phylogenetic relationship of Monogenea from Cichlidae (shaded rectangles) and other host groups. As branch support, Bremer support values are shown. Geographic distribution of species of each genus is depicted above generic names ("Mar.": marine; "Mad.": Madagascar; "S. Am.": South America).

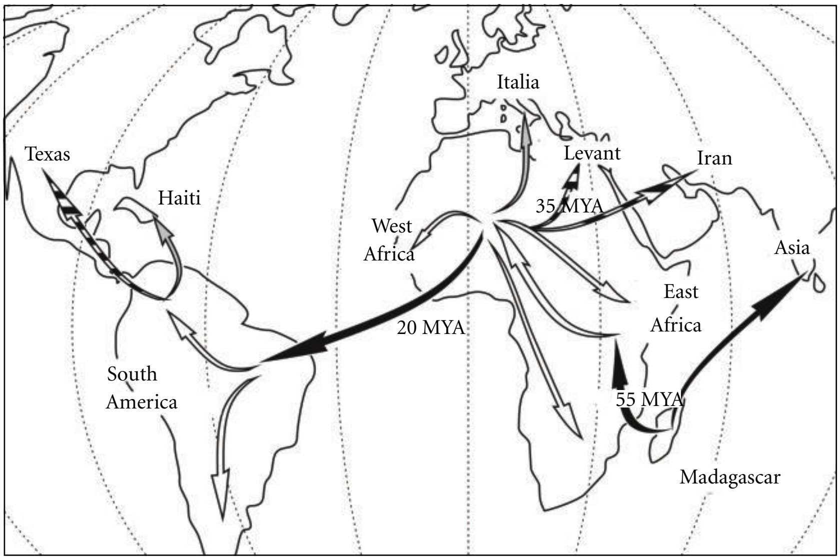


FIGURE 3: Simplification of Murray's hypothesis [25] on the origin and biogeography of the world's cichlids. Arrows symbolising dispersal routes are indicated as follows: marine (black); freshwater (white); unresolved (black and white); unknown (fossils only; grey).

5.2. Lessons from the Cladistic Analysis. The morphology of Monogenea and other flatworms is prone to display homoplasy, suggesting molecular phylogenetics to be needed to conclusively resolve their evolutionary relationships [81, 136, 137]. Indeed, plasticity has been shown both in haptoral [138–142] and genital [143–145] structures. This demonstrates that not only phylogeny but also geography, host-related and environmental factors, and (sexual) selection may influence the morphology of monogenean hard parts. On the other hand, most of this variability is continuous/quantitative and between conspecific individuals, and, hence, should not represent a significant interference in construction of phylogenetic hypotheses based on discrete characters differing between genera. Moreover, ambiguities or resolution problems related to morphological phylogenies were mostly shown in groups of organisms displaying taxonomic difficulties and a limited number of discretely varying haptoral characters (e.g., gyrodactylid Monogenea [137]), lacking sufficient characters comparable throughout the family (e.g., six in capsalid Monogenea [81]), or where plastic characters, prone to loss or acquisition, were used (e.g., mouthpart structure reflecting trophic adaptation in lysianassoid Amphipoda [146]). In contrast, based on a limited number of species of ancyrocephalid Dactylogyridea, there are clear indications that haptor morphology contains phylogenetic signal [107, 108]. On the level within or between genera, haptoral (and, on the between-species level: genital) structure does not conflict with molecular taxon boundaries and is systematically informative. Indeed, on these levels, geography or host characteristics do not seem to influence haptor morphology in this monogenean family.

The only genetic data on monogeneans infecting cichlid fishes stem from African species [100, 107], and are therefore uninformative for intercontinental comparison. Hence, a morphological phylogenetic hypothesis, based on a combination of haptoral and genital elements, seems a reasonable approach given the currently available knowledge. As any scientific hypothesis, it is prone to extensive reconsideration once sufficient genetic data are collected, as such data will yield a higher number of informative characters [147] compared to the presently available morphological knowledge. The morphological phylogenetic hypothesis (Figure 2) is fundamentally compatible with the results of the molecular phylogeny proposed by Mendlová et al. [100], with some more conspicuous coincidences (e.g., on the sistergroup relation of *Scutogyrus* and *Cichlidogyrus*). Also the close affinity between *Ceylonotrema* and *Diplectanum* might merit further scrutiny, as the ventral and dorsal bars of *Ceylonotrema* indeed show similarities to those in diplectanids. As molecular phylogenetics also faces, among others, problems with homoplasy [148–151], combining insights from both phenotypic and genetic classification in testing hypotheses and identifying uncertainties seems the most fruitful way forward (e.g., Rota-Stabelli et al. [152] for deep phylogeny of arthropods).

Although the above-mentioned flaws limit the interpretation we can give to our tree reconstruction in Figure 2, which should not be regarded as a definitive hypothesis on dactylogyridean evolutionary history, this tree provides the first comprehensive interpretation on phylogenetic relationships among cichlid monogeneans. Although based on morphological characters, it represents a phylogenetic hypothesis that may be tested based on molecular data. Furthermore, the CIs and RI of the tree demonstrate the existence of phylogenetic signal, independent of the number of homologous series available.

The cladogram indicates that parasitizing on cichlids is a polyphyletic character in Dactylogyridea. The non-sistergroup relationship of the ectoparasitic Dactylogyridea of cichlids on the different continents seems an interesting argument to support the dispersal hypothesis. On the other hand, if the parasites distribution is to be explained mainly by oceanic dispersal (of their hosts), one would expect their phylogenetic relationships to follow continental borders (as suggested for Gyrodactylidae by Boeger et al. [95]). The ectoparasite genera of different continents, however, are not each other's sister taxa based on the morphological cladogram (Figure 2). Hence, host-switching events from other fish groups are required, under whatever scenario, to explain the host-parasite distribution observed in the preliminary cladogram. Therefore, the evolutionary history of other fish families serving as "source hosts" should be considered.

Because Monogenea with a marine lifestyle appear as sister taxa to several cichlid (and thus, freshwater) parasites in our cladogram, lateral transfer from other (including marine, necessitating freshwater/marine transition) hosts could be needed to explain our cladogram, irrespective of the cichlid scenario (Figure 2). Host-switching [153], even between fish hosts that differ at the ordinal level [154], as well as substantial salinity tolerance within species [155] have been observed in Monogenea. Although both have mainly been demonstrated in gyrodactylids, even representatives of the ancyrocephalid *Cichlidogyrus* were suggested to colonize Cyprinodontiformes as a result of ecological transfer from cichlid hosts [156]. Transfer of marine parasites to cichlids is proposed by Pérez-Ponce de Léon and Choudhury [59] to explain the acquisition of additional parasites (cryptogonimid Digenea) after the cichlid's colonization of Mexico. The authors invoke the salinity tolerance of those cichlids, allowing ecological contact with marine or estuarine fishes and, hence, host-switch events. Another example, for cichlids cultured under marine conditions, is provided by Kaneko et al. [123], reporting the acquisition of the marine capsalid monogeneans *Neobenedenia melleni* (MacCallum, 1927) and *Benedenia monticelli* (Parona and Perugia, 1895) by *Oreochromis mossambicus* (Peters, 1852) and *O. aureus* (Steindachner, 1864), respectively. Those authors mention less-than-optimal adaptation to marine conditions as cause for the susceptibility of the cichlids to a parasite not normally adapted to those hosts, although *N. melleni* has a rather wide host range anyway.

Hence, freshwater/marine transfers are possible and a marine environment lowering the cichlid's condition could ease the colonization by parasites not specialised in cichlids. In favour of the dispersal hypothesis, one could argue that marine migration, freeing cichlids of other ectoparasitic Monogenea and hence removing interspecific competition, would be an additional facilitating factor for ecological transfer. However, it is often assumed that interspecific competition is not such an issue in Monogenea [157–159]. Freshwater/marine transitions would seem to contradict the above-mentioned limited tolerance to osmotic shocks. However, those ecological transfers across salinity borders should be thought of mostly on an evolutionary timescale, and as a feature occurring in certain genera with a broad salinity tolerance ([100] for *Protogyrodactylus*), rather than as frequent events in a monogenean lifespan.

5.3. A Putative Scenario Assuming the Dispersal Hypothesis.

The Gondwanan vicariance hypothesis implies isolation of fish populations remaining in their respective freshwater environments. In contrast, in case the present-day distribution pattern of the radiation of cichlids would be the result of dispersal, the hypothesis of Murray [25] applied to their parasites gives us the following scenario. We do not mean to say that the current evidence unequivocally points in this direction; we only outline this scenario as a “thought experiment” as it could explain the present continental differences between cichlid fish parasites. It should also be clear that, whatever the nature of the intercontinental migrations, the present-day cichlid distribution is a result of both vicariance and dispersal. For instance, intracontinental migrations (through freshwater systems) have happened in either case, and have caused the ectoparasites to display a substantial similarity across the respective continents.

(1) If Madagascar is indeed the centre of origin of Cichlidae, *Insulacleidus* probably represents the only extant representative of the most basal clade of ectoparasitic Monogenea associated to this fish family. In view of the very simple morphology of its haptoral hard parts, this seems an acceptable assumption.

(2) Two marine migrations from this island took place in the late Cretaceous (50–55 MYA [25]), one towards Asia, and another one towards Africa, leading to putative loss of all Malagasy ectoparasites and subsequent infection by parasites of different host families in the new continent. This created the current differences in ectoparasitic fauna between the continents (Figure 1). Mesoparasites, however, were retained during marine migrations. The difference observed for these parasites (*Enterogyrus* spp.) is the result of simple isolation-by-distance. If this were the case, no reproduction would have occurred (or been required) during the migration. Salinity would have posed an obstacle to reinfection during the migration because monogenean larvae (oncomiracidia) are free-living organisms and would not survive this salinity change. The retention of mesoparasites would have been possible, however, if the migrations occurred quickly, allowing the individual adult parasites of migrating hosts to survive the migration. It is important to note that *Enterogyrus*, just like *Urogyrus*, is only known from cichlid hosts [78]. There is, hence, no reason to assume that the presence of representatives of this genus on different continents is a consequence of host-switch from other hosts.

(3) A second migration through marine waters took place between West Africa and South America (20 MYA [25]), with the same consequences as above: loss of African ectoparasites, infestation of newly arrived “clean” hosts by South American ectoparasites coming from other host families. The time for migration (from Africa to America) was estimated (based on palaeoreconstructions and with dispersal through the aid of oceanic currents and shallow water areas) at 23 days [25], which is compatible with the survival of mesoparasites without reproduction (*cfr. supra*). This implies that *Enterogyrus*, or a closely related sister clade, might be present in South American cichlids (the only observation of this genus in the Americas is from an introduced African species [117]). Furthermore, even without having to invoke open-oceanic migration, northern landbridges and stretches of less saline seas provided dispersal pathways for freshwater fishes between Europe and the Americas during the late Cretaceous and Tertiary [13]. Thus, the cichlid fossils of Europe ([25]; *cfr. infra*) could fit well in this hypothesis. Also, the still ongoing debate on whether the salinity tolerance of cichlids suffices for oceanic migration would pose no problem here, as dispersal over landbridges or through diluted marine or coastal environments would not require them to withstand marine open-water conditions. In fact, it seems harder to find a plausible dispersal path for cichlids towards Asia. In contrast, for Mastacembelidae, a fish family with an African-Asian distribution where palaeontological and molecular evidence are in favour of dispersal rather than vicariance, a land bridge (over the Middle East) rather than marine migration is suggested as pathway [160]. The Middle East is also known as a centre of exchange for other freshwater fishes, for example, in cyprinids [161].

(4) Intracontinental migrations lead to the wider colonization of Africa and South America. As these migrations occurred only in freshwaters, there were no losses of parasites, and the parasite fauna now observed is remarkably homogeneous. Mendoza-Franco and Vidal-Martínez [162] propose an example of this, in *Sciadicleithrum*, which would have migrated with its cichlid hosts from South to Central America after the uplift of the Panama Isthmus. An exception for Africa is *Onchobdella*, only infecting hosts with a distribution limited to West Africa (though its mesoparasitic putative sister *Urogyrus* is more widespread in the continent, *cfr. supra*). In view of Figure 2, their ancestor probably colonized cichlids after lateral transfer from another fish host family.

(5) During the African or American intracontinental expansion of the Cichlidae, three lineages isolate themselves: one colonized the Levant (35 MYA [25]), another one Iran, and the last one North America (Rio Grande river, where *Herichthys cyanoguttatus* Baird and Girard, 1854 presently occurs [163]).
In her paper, Murray [25] does not conclude whether those fishes migrated through marine or freshwater systems (Figure 3, black and white striped arrows). As Levantine cichlid parasites (ecto- and meso-) are similar to African ones, we suppose that the migrations which introduced this fish family to this area solely involved crossing freshwaters (for otherwise, the ectoparasitic fauna should be different). While Werner and Mokady [164] suggest much more recent colonisation for the only Levantine haplochromine, *Astatotilapia flaviijosephi* (Lortet, 1883), they do not

invoke marine dispersal for its arrival from Africa into the Levant either.

For the Iranian or North American colonization, we should be able to assess the likelihood of marine or freshwater migration by describing ectoparasites from those cichlid species. Ancyrocephalidae have hitherto not been found on them; only 20 specimens, provided by B. Jalali, of *Iranocichla hormuzensis* have been studied, yielding only gyrodactylid monogeneans (A. Pariselle, unpublished data). No data were available for Rio Grande fishes. In both cases (Iran and North America) *Enterogyrus* (or a related genus—being a mesoparasite) should be present.

(6) The presence of cichlid fossils is demonstrated in Europe (Italy) and Central America (Haiti). As these faunas are extinct, their parasites will remain unknown and their migration pathways cannot be inferred from host-parasite data (Figure 3, grey arrows).

6. Conclusions and Suggested Approach

The current knowledge on diversity and distribution of parasites does not allow us to be conclusive in supporting either the dispersal or vicariance hypothesis explaining the present-day distribution of cichlids. The interesting outcome of analysing the parasite data is that the resulting conclusions may be tested and falsified. Although the presence of *Enterogyrus* on cichlid hosts worldwide (i.e., in South America and/or Madagascar) has not been demonstrated yet, the most crucial argument here could be provided by a sound phylogeny of dactylogyridean ectoparasites of cichlids (for which the data are currently lacking). In view of classical problems in molecular phylogenetic reconstruction, such as introgression [165] and discordance between conclusions based on mitochondrial *versus* nuclear markers [166], multigene approaches are recommended. One should examine, using molecular phylogenetics, whether there consistently is a closer relationship between Asian, African, or South American cichlid ectoparasites with other ectoparasites from different local host families, than with the parasites of cichlid hosts on other continents. Apart from sistergroup relations, genetics could also give us clues on the evolutionary distances between the various cichlid monogeneans. This is crucial, as the mesoparasite *Enterogyrus* could have a slower rate of evolution and diversification than the various ectoparasitic genera (perhaps due to high constraints resulting from its mesoparasitic lifestyle), possibly explaining the higher diversity in ecto- than in mesoparasites. Though little molecular evolutionary data exist of these animals, results of Mendlová et al. [100] do not support this hypothesis. Moreover, ectoparasitic Monogenea may depend for their speciation on their hosts' diversification ([167] but see [168]), making a speciation burst independent of cichlid history unlikely. Furthermore, mesoparasitism is a derived and polyphyletic feature in our cladogram (and a derived character in the phylogeny of Mendlová et al. [100]), so there is little reason to assume that those parasites in general evolve in a different way from their ectoparasitic counterparts.

Finally, we hope to have exemplified that the evolution and biogeography of parasites should be considered in association with a sound knowledge of their hosts. Indeed, Murray's theory on age and dispersal pattern of cichlids could be in agreement with their continent-specific fauna of ectoparasitic Monogenea, though extra (molecular) data are evidently needed to be conclusive. Conversely, we have to keep in mind the contribution that the study of parasites can make to investigations concerning their hosts, at different levels. This spans from the most specific, where parasites can assist in the identification of host sister species [74, 169], up to the broadest, as in the example presented here, where parasites might lead us to choose between two hypotheses on host origin. On an intermediate level, parasites might resolve an ambiguity on the mode of cichlid biogeographical evolution: for example, that the Levantine migration occurred by crossing freshwater rather than marine systems.

Appendix

(1) Hook: shank tapering proximally; shank with bulb at proximal end. (2) Hooks: similar in shape and size; hook 5 almost splinter-like; greatly variable in size. (3) Thumbs of hooks: erected; straight; depressed. (4) Number of distinct portions in hook shank: one; two. (5) Ventral anchor: fully developed; splinter-like; one fully developed, another splinter-like. (6) Ventral bar—anterior margin: without obvious ornamentation; with subterminal flaps; with large shield-like plate; with small flap. (7) Ventral bar shape: straight; V-shaped; inverted V-shaped; arched. (8) Longitudinal groove on ventral bar: absent; present. (9) Dorsal bar: single; double; absent. (10) Dorsal bar—subterminal anterior flaps: absent; present, well developed and supported by ridges; present, no supporting ridges. (11) Dorsal bar—anterior auricles: absent; present. (12) Dorsal bar: straight or slightly V-shaped; M-shaped; inverted V-shaped. (13) Accessory piece: present; absent. (14) Male copulatory organ: coiled; somewhat straight or straight. (15) Articulation of copulatory complex: articulated; nonarticulated. (16) Vagina: ventral; dextrolateral; sinistrolateral. (17) Vagina: sclerotized; nonsclerotized.

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