

Evolutionary divergence in replicate pairs of ecotypes of Lake Victoria cichlid fish

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ABSTRACT

Questions: (1) Do replicate pairs of ecotypes of cichlid fish represent different stages of ecological speciation? (2) Are phenotypic and genetic divergence correlated with each other and with the steepness of the habitat gradients?

Study system: Three replicate pairs of putative ecotypes of cichlid fish in the genus *Neochromis* from three islands in Lake Victoria. The three pairs present similar trophic polymorphisms. The three islands differ in steepness of the benthic habitat gradients mediated by variation in water clarity, shore slopes, and depths of the rock–sand interface.

Analytical methods: We quantified fish body morphology and dentition, typed population samples at nine microsatellite loci, and analysed how phenotypic and neutral genetic variation were distributed among ecotypes and along the habitat gradients.

Results: Despite weak or absent genetic differentiation at neutral markers, ecotypes were divergent in phenotypes in a replicated manner, involving from one to many different traits in a nested series. Variation in eco-morphological traits and allelic variation at neutral marker loci were associated with depth of habitat at some islands.

Keywords: divergent selection, F_{ST} , speciation, trophic polymorphism.

INTRODUCTION

Divergent selection between habitats or niches, resulting from the interaction of individuals with their biotic and abiotic environment, is thought to be a common mechanism of population divergence and speciation (Schluter, 2000; Rundle and Nosil, 2005; Maan and Seehausen, 2011). Spatially coincident habitat and resource structure (i.e. coincidence of alpha and beta niche) is thought to facilitate this process (Schluter, 2000; Via, 2001). When mating and feeding occur in

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the same habitat, adaptation along a habitat gradient will cause a reduction of gene flow along the gradient as a by-product (Endler, 1977). More recent models of speciation on habitat gradients suggest that local competition for resources, together with divergent selection along the gradient, can initiate ecological character displacement between diverging populations and complete speciation (Case and Taper, 2000; Doebeli and Dieckmann, 2003; Leimar *et al.*, 2008). The spatial scale at which this may or may not occur is, however, still a matter of debate (Coyne and Orr, 2004; Bolnick and Fitzpatrick, 2007). The strength of selection versus the extent of dispersal and gene flow between demes along the gradient are expected to predict the magnitude of the adaptive divergence and the likelihood of ecological speciation (Doebeli and Dieckmann, 2003; Gavrillets, 2004; Kawata *et al.*, 2007; Hendry, 2009). Specifically, gradients promoting some direct ecological interaction between divergently adapted phenotypes may increase the effectiveness of selection over those where there is no interaction between phenotypes with divergent adaptation. This is because in the first case negative frequency-dependent disruptive selection may arise from local competition, facilitating local coexistence of divergent phenotypes and the evolution of assortative mating through a reinforcement-like process (Doebeli and Dieckmann, 2003; Gavrillets, 2004). However, large amounts of gene flow can easily cancel the effects of selection and constrain adaptive divergence (Rasanen and Hendry, 2008). Empirical studies of natural replicates of ecotypic divergence of variable completeness from similar starting conditions are useful means of investigating the spatial context promoting or inhibiting phenotypic differentiation and speciation (Seehausen *et al.*, 2008; Berner *et al.*, 2009; Nosil *et al.*, 2009; Renaut *et al.*, 2011; Kaueffer *et al.*, 2012).

Known for their outstanding diversity in morphology, behaviour, and coloration, rock-dwelling cichlid fish of Lake Victoria are useful systems for the study of divergence along environmental gradients. These fish live on isolated rocky patches along the coast and around offshore islands varying in important environmental factors such as water clarity, light penetration, rocky substrate structure, and inclination of the shore slope. Variation in the steepness of habitat gradients generated by variation in water clarity and shore slope has been shown to influence the extent of species differentiation in some taxa (Seehausen *et al.*, 1997, 2008). The majority of sympatric and parapatric sister species pairs of Lake Victoria cichlids show very different breeding coloration but little morphological differentiation, and many species display conspicuous polymorphisms in coloration (Seehausen and Van Alphen, 1999; Seehausen *et al.*, 1999; Seehausen and Schluter, 2004; Magalhaes *et al.*, 2009, 2010). Hence more studies have focused on the origin and maintenance of colour polymorphisms and their role in speciation than on the role of other ecologically relevant polymorphisms. Distinct polymorphisms in trophic morphology are scarce in Lake Victoria cichlids, but a few cases have been identified (Seehausen, 1996; Seehausen *et al.*, 1998). In this study, we investigated three replicates of a trophic polymorphism first described 16 years ago (Seehausen, 1996), from three islands with different habitat structure and variable degrees of ecotypic differentiation.

The polymorphism was reported within three species of the genus *Neochromis* – rock-dwelling, predominantly algae-scraping cichlids, widely distributed around rocky islands in Lake Victoria. The three cases, composed of two ecotypes each, appear to follow a repetitive pattern: one ecotype is a typical algae scraper with bicuspid teeth in the outer tooth row, a deep body, and steeply decurved dorsal head profile, whereas the other is more slender bodied, with unicuspid teeth in the outer tooth row (Fig. 1; see Methods and materials for details on the study species). The islands are separated by large habitat discontinuities (many kilometres of deep water with a sandy bottom over which *Neochromis* are never found). The shores of the three islands differ in water clarity, steepness of the

slope, substrate, and depth of the rock–sand interface. All these environmental variables influence the distribution of resources and the potential distribution of the fish, making inferences about which ones are important for differentiation difficult in an analysis of a relatively small number of islands. Nonetheless, it allowed us to evaluate the importance of depth gradients as axes of eco-morphological and neutral genetic differentiation.

To explore the extent of ecotypic differentiation, we analysed how variation in morphology, dentition, and nine putatively neutral microsatellite markers was partitioned between the two ecotypes at each island. Significant differences in eco-morphology would support the idea of divergent ecological selection. Differentiation at neutral genetic markers would suggest the evolution of generalized barriers to gene flow and speciation.

We tested the hypothesis of formation of clines in feeding-related characters and in neutral genetic markers over water depth by correlating morphology, dentition, and neutral genetic markers with depth at capture of individuals. Significant correlations would support the role of depth gradients as important axes of eco-morphological and genetic differentiation.

In addition, to determine whether – in the absence of differentiation at neutral genetic markers at Bihiru Island (see Results) – differences in body morphology and dentition could be entirely due to phenotypic plasticity, we performed a common garden experiment using the ecotypes of *Neochromis* sp. ‘Bihiru scraper’.

METHODS AND MATERIALS

Study species: geographical distribution and description of the polymorphisms

Neochromis omnicaeruleus (Seehausen and Bouton, 1998) and *Neochromis greenwoodi* (Seehausen and Bouton, 1998) are two species of rock-dwelling algae scrapers widely distributed in Lake Victoria (Fig. 1). They are allopatric sister species with complementary and adjacent distributions. Slender built *Neochromis* with mostly unicuspid teeth, collectively referred to as *Neochromis* sp. ‘unicuspid scraper’, are known from four locations. At two of these they coexist with *N. greenwoodi* (Igombe Island, Bwiru Point (rare)) and at two others with *N. omnicaeruleus* (Makobe Island, Ndurwa Point (rare)). *Neochromis* sp. ‘Bihiru scraper’ is an undescribed distinct species known only from Bihiru Island. *Neochromis greenwoodi* also occurs at this island; however, it is rare there and phenotypically very distinct from *Neochromis* sp. ‘Bihiru scraper’.

Neochromis omnicaeruleus, sampled for this study at Makobe Island, is a typical algae scraper with a relatively large head, deep body, and specialized dentition of densely spaced bicuspid teeth in the outer tooth rows and many inner tooth rows (Seehausen *et al.*, 1998). Males have characteristic bright metallic blue or yellow nuptial coloration while females of the most common colour morph are yellow-brown. Both sexes have dark vertical bars. Abundances of this species are highest at depths of between 1 and 3 m but it has been found deeper than that (Seehausen and Bouton, 1997), effectively at the depth at which the rocky habitat gives way to sand. *Neochromis greenwoodi*, sampled at Igombe Island, is ecologically similar to *N. omnicaeruleus* but has a typical male breeding coloration that is uniformly black with a narrow red caudal fin edge while females are typically dark brown. Its depth distribution is similar to that of *N. omnicaeruleus*.

The name of *Neochromis* sp. ‘unicuspid scraper’ (Seehausen, 1996) derives from the fact that adult fish have widely spaced unicuspid teeth. This type of dentition is uncommon, and

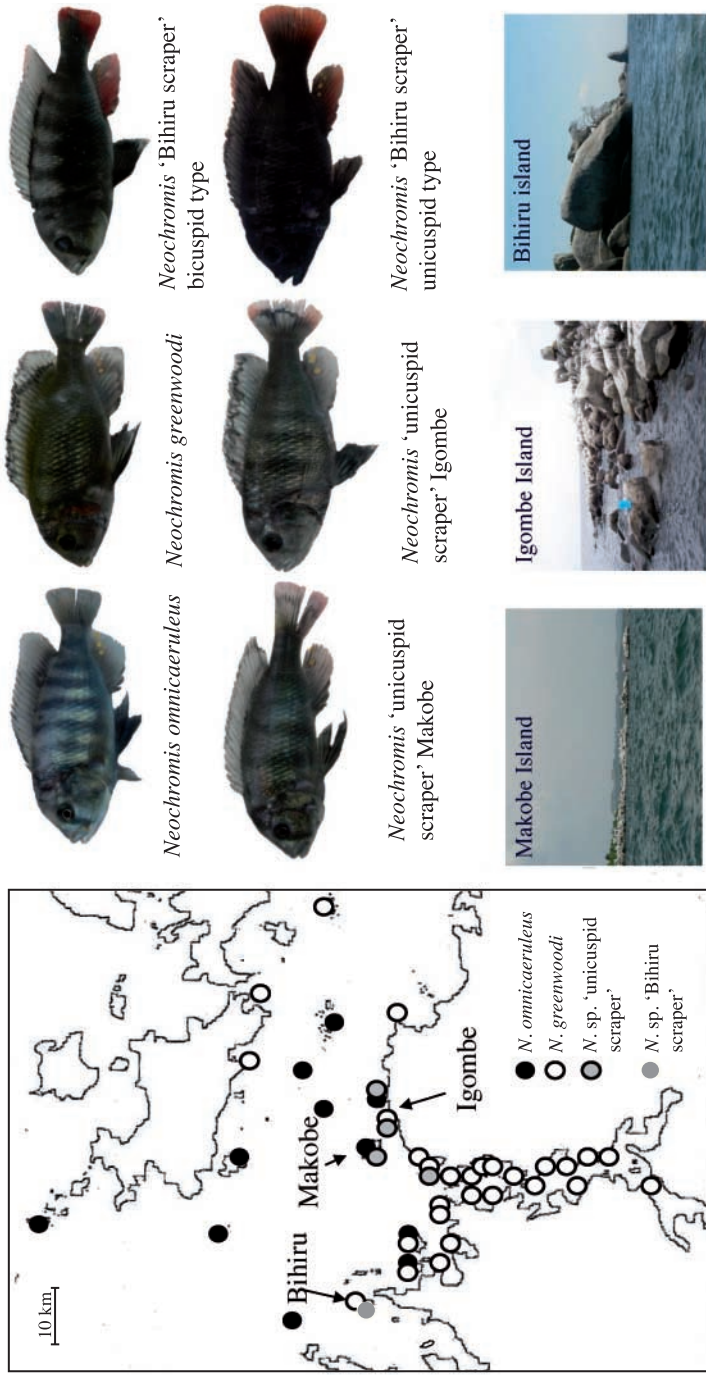


Fig. 1. The ecotypes and their geographic distributions. Map of the southern part of Lake Victoria showing known distributions of the species (adapted from Seehausen, 1996). Arrows indicate the three sampling locations: Makobe, Igombe, and Bihiru islands. On the right of the map are pictures of the shores of each island and of the ecotypes studied at each location.

otherwise known within the *Neochromis* genus only in the unicuspid morph of *Neochromis* sp. 'Bihiru scraper'. *Neochromis* sp. 'unicuspid scraper' have a slender body and a dark blue-grey breeding coloration. They inhabit slightly deeper regions than the typical algae-scraping *N. omnicaeruleus* and *N. greenwoodi* on average, and can be observed at the interface between rock and sand down to at least a depth of 8 m.

Neochromis sp. 'Bihiru scraper', only known from Bihiru Island (Seehausen, 1996), inhabits a range of microhabitats and a depth range from less than 2 m to about 9 m. It was suggested to exhibit a fairly discrete polymorphism, with two trophic morphs that are characterized by different tooth morphology, associated with subtle differences in body and jaw shape but no consistent differences in male coloration (Seehausen, 1996). However, none of these traits had been quantified and the existence of two discrete ecotypes as opposed to a continuously variable population remains to be confirmed.

In the figures and tables, we will refer to *N. omnicaeruleus*, *N. greenwoodi*, *Neochromis* sp. 'unicuspid scraper' from Makobe and Igombe, and *Neochromis* sp. 'Bihiru scraper' bicuspid morph and unicuspid morph as N.o., N.g., N.us Ma, N.us Ig, N.Bs bic, and N.Bs uni, respectively. In the remainder of the paper, we refer to the two morphs or species within each island simply as 'ecotypes'.

Environmental gradients

Water depth and exposure of substrate to sunlight strongly affect the abundance and composition of food resources on the rocks, and the sloping lake floor therefore constitutes an important dietary resource gradient. High water clarity and a gently sloping floor make the transition from sunlight-rich, primary-production-dominated shallow water to less well illuminated, deeper water, long and gradual. Small boulders generate a locally homogeneous light environment and a spatially linear gradient of light-dependent resources, whereas large boulders create a mosaic of light and shadow. Each of the islands studied has a different combination of water clarity, shore slope steepness, and boulder size (Table 1), generating between-island variation in steepness and in spatial linearity of environmental gradients. Makobe Island has the most gently sloping shore and the highest water transparency, which together create the shallowest environmental gradient of the three islands. The shore of Igombe Island is in many ways similar to that of Makobe but has a steeper environmental gradient (Seehausen *et al.*, 1997). This island has a steeper shore slope, the rock-sand interface is at a lesser depth, and water transparency is lower. Finally, the

Table 1. Geographical and habitat information and number of individuals (*N*) collected for each island

Island	Latitude	Longitude	Shoreline slope	Rock-sand interface (m)	Secchi depth (cm)	<i>N</i>
Makobe	02°22'S	32°39'E	6.6°	5-7	287	65
Igombe	02°23'S	32°37'E	17.9°	4-5	216	47
Bihiru	02°22'S	32°55'E	36.8°	4-9	248	62

Note: The Secchi depth is the average Secchi depth measured over several sampling days. This was always done in the morning between 08.00 and 09.00 h. The slope of the shore was estimated by the equation (depth 10 m offshore [cm]/1000) × 45°. The rock-sand interface was estimated by registering the depth in metres at which rocks became scarce and sand was the most common substrate while scuba-diving using a diving computer.

shore of Bihiru Island is extremely steep, which, despite the clarity of the water, creates a steep environmental gradient.

Sampling

Rock-dwelling cichlids are sexually dimorphic; females are smaller than males and tend to be cryptically coloured, making identification to species level among very closely related species more difficult than for males. Therefore, we used only males for this study. We targeted adults because eco-morphological traits, including tooth shape, are subject to ontogenetic change. Immediately after capture, we photographed every male in a custom-made Perspex photographic cuvette. Identification of individuals to ecotype was done by visual inspection of individual photographs, using a combination of head and body profile at Bihiru, and head and body profile and male nuptial coloration at Igombe and Makobe. We neither took morphological measurements nor analysed dentition and genetics before the identification of individuals to an ecotype.

Through angling and gill-netting we collected a total of 174 males at the three islands during October and November 2005: 45 *N. omnicaeruleus* and 20 *Neochromis* sp. ‘unicuspid scraper’ at Makobe Island, 30 *N. greenwoodi* and 17 *Neochromis* sp. ‘unicuspid scraper’ at Igombe Island, and 62 *Neochromis* sp. ‘Bihiru scraper’ (29 assigned to the bicuspid ecotype and 33 to the unicuspid ecotype) at Bihiru. After humanely killing the fish by overdosing with an anaesthetic, we took fin clips for genetic analysis and the specimens were fixed in 4% formaldehyde solution and later transferred to ethanol for storage. For each individual we recorded the water depth at which they were collected by measuring the length of the angling line between bottom and surface or recording the depth at which nets were set.

Analysis of phenotypic variation and divergence

To analyse morphology, we measured 13 standard morphometric distances using a digital calliper (Barel *et al.*, 1977): standard length, body depth, head width, head length, snout width and length, lower jaw width and length, eye depth and width, cheek depth, pre-orbital depth and inter-orbital length (see Appendix Fig. S1; for all supplementary material in this paper, go to www.evolutionary-ecology.com/data/2739Appendix.pdf) and log transformed all variables. To adjust each morphometric measurement for size heterogeneity among individuals, we performed pooled within-groups regressions of all log-transformed variables against the log-transformed standard length after checking for and finding parallel slopes among groups (Thorpe, 1976; Fleming and Gross, 1994). We subsequently used the residuals from these regressions in a principal components analysis to reduce the number of distances to independent variables. We extracted principal components (PCs) from covariance matrices and we retained the first three axes, which we then used in all further analyses.

Haplochromine oral tooth shape is subject to ontogenetic changes. Sub-adult Lake Victoria haplochromines generally have bicuspid teeth that change to weakly bicuspid and unicuspid through a gradual reduction in size of the minor cusp and prolongation of the major cusp. The extent to which this happens varies between species and sexes within species. In most species of *Neochromis*, the teeth remain bicuspid; however, this isn’t the case in the unicuspid ecotypes we studied here, where teeth often turn nearly or entirely unicuspid. The upper jaw teeth are arranged in multiple rows, with algae scrapers having typically a large number of rows. For each individual, we counted the number of unicuspid,

bicuspid, and tricuspid teeth in the outermost tooth row of the upper jaw and the number of tooth rows in the upper jaw. We calculated the percentage of unicuspid teeth by dividing the number of unicuspid teeth in the outermost row of the upper jaw by the total number of teeth in the same row.

To provide visual representations of trait distributions, we constructed density plots of the first three PC scores and of the percentage of unicuspid teeth for each island separately using 'mclust' (Fraley and Raftery, 2006). For the number of tooth rows, we plotted histograms.

To analyse how differences in morphology and dentition are partitioned between the three islands and the two ecotypes at each island, we performed nested univariate analyses of variance (ANOVAs) with a Gaussian distribution on the percentage of unicuspid teeth and on the first three morphological PCs [two factors: ecotypes (2 ecotypes) nested in islands (3 islands)]. For number of tooth rows, we performed a similar analysis (nested ANOVA) with a Poisson distribution. For traits showing significant differences between ecotypes, we then performed Tukey *post-hoc* tests to further investigate which groups were significantly different.

Finally, to test for genetic/developmental non-independence of the traits analysed, which is important for the interpretation of our results, we performed Pearson correlations between number of rows, percentage of unicuspid teeth, and PC scores for each species separately.

Analysis of neutral genetic variation and divergence

We extracted DNA from the fin tissue of 174 individuals using a QIAGEN® (Basel, Switzerland) Biosprint™ 96 extraction robot with a corresponding standard digestion protocol. We used nine microsatellite loci developed for Lake Victoria cichlids [Ppun 5, Ppun7, Ppun17, Ppun21, and Ppun32 (Taylor *et al.*, 2002)] and for other African cichlids [OSU20d, OSU19T, OSU16d (Wu *et al.*, 1999), and TmoM5 (Zardoya *et al.*, 1996)] (for details on microsatellite amplification, see www.evolutionary-ecology.com/data/2739Appendix.pdf).

We used CONVERT (Glaubitz, 2004) to create input files for other programs and to create allele frequency tables. We checked genotypes for scoring errors that might be attributable to stutter products, large allele dropout, or the presence of null alleles, using MICRO-CHECKER v.2.2 (Van Oosterhout *et al.*, 2004). We used ARLEQUIN v.3.11 (Excoffier *et al.*, 2005) to test each locus in each species for departure from Hardy-Weinberg equilibrium, to calculate observed (H_O) and expected (H_E) heterozygosities, and to perform tests for linkage disequilibrium (LD). We estimated the multilocus inbreeding coefficient (F_{IS}), H_O , and H_E for each group using Genetix v.4.05 (Belkhir *et al.*, 1996). We adjusted the statistical significance in the above tests for multiple comparisons using a false discovery rate (FDR) of 0.05 (Benjamini and Hochberg, 1995).

To determine whether ecotypes within islands were genetically most similar, we constructed an ecotype tree based on genetic distances in PHYLIP v.3.69 (Felsenstein, 2009). The Neighbour-Joining tree based on F_{ST} values was constructed using the PHYLIP software package. Bootstrap values of 1000 replicates were obtained to test the robustness of the tree topology. We used MEGA 5 (Tamura *et al.*, 2011) to draw the final tree.

To visualize the partitioning of molecular variance among individuals within and between islands, we performed a factorial correspondence analysis (FCA) over individual allele frequencies in Genetix v.4.05 (Belkhir *et al.*, 1996).

We performed model-based assignment tests on genotypes from all islands combined, as implemented in the program STRUCTURE v.2.2 (Pritchard *et al.*, 2000). Markov Chain

Monte Carlo (MCMC) simulations were run with 500,000 replicates and a burn-in of 50,000 replicates for K (number of populations) = 1 to 6 and applying the admixture model.

To account for the probability that individuals are migrants from other, sampled or unsampled populations, we used GENECLASS v.2.0 (Cornuet *et al.*, 1999). We selected the detection of first-generation migrants, which identifies individuals not born in the population where they were sampled. We estimated the 'L_home', the likelihood of finding a given individual in the population in which it was sampled, which is the most appropriate estimation to use when not all potential source populations have been sampled (Paetkau *et al.*, 2004). We used a frequencies-based method (Paetkau *et al.*, 1995) and Monte-Carlo resampling of 10,000 individuals per locality (Paetkau *et al.*, 2004).

As measures of neutral genetic differentiation, we used single-locus and multi-locus F_{ST} values and the corresponding 95% confidence intervals, which were estimated using ARLEQUIN v.3.11 (Excoffier *et al.*, 2005). The sorting of individuals into ecotypes by external morphology is a conservative method when asking if ecotypes differ in dentition characteristics, but if tooth shape is their most strongly divergent trait, it may underestimate neutral genetic differentiation between morphs because it is likely to contain some identification error. As density plots for the percentage of unicuspid teeth revealed two distributions with a trough around 50% at each island, we also calculated the F_{ST} values between the group of individuals with more than 50% unicuspid teeth and that with less than 50% unicuspid teeth for each island after quantification of their dentition.

The distribution of phenotypic and genetic variation over water depth

We used Mann-Whitney tests to test the null hypothesis that ecotypes had the same depth distribution within each island. In addition, we calculated Spearman-rank correlations between capture depth and number of tooth rows, percentage of unicuspid teeth, and the first three morphometric PCs.

To test for effects of water depth on neutral genetic variation, we used an individual-based approach. Matrices of capture depth differences and genetic distances (\hat{a}) (Rousset, 2000) between individuals were computed for each island separately using the program SPAGeDI (Hardy and Vekemans, 2002). We then tested the significance of relationships between the two matrices with a Mantel test based on 1000 permutations as implemented in ARLEQUIN (Excoffier *et al.*, 2005).

Common garden experiment and analysis of laboratory-bred fish

To test if the phenotypic differences between ecotypes observed in the wild were entirely due to phenotypic plasticity, we performed a common garden experiment using *Neochromis* sp. 'Bihiru scraper', for which we found no neutral genetic differentiation between ecotypes (see Results).

We performed the experiment using as prospective breeders 15 males from the two ecotypes and 9 females collected at Bihiru Island in November 2005 (for details on the common garden experiment, see www.evolutionary-ecology.com/data/2739Appendix.pdf). We produced six F1 families from three different mothers and six different fathers. So we had three unrelated half-sib families that did not share either mothers or fathers, each

composed of two full-sib families that shared the same mother but not the father. One full-sib family within each half-sib family had a father of the unicuspid ecotype, while the other one had a father of the bicuspid ecotype. We raised each full-sib family in a tank on its own. Tanks were randomized within the breeding room. Tooth shape polymorphism in *Neochromis* sp. 'Bihiru scraper' is only clearly apparent in males (Seehausen, 1996), so we analysed only the males' phenotypes in each family. We analysed a total of 40 males that were over 12 months old from the six families (3–19 males per family).

Parental individuals were not available for phenotypic analysis because, to complete the breeding scheme, we could not preserve them before they showed phenotypic effects of ageing. To test for family effects on the phenotypic variation observed among the experimental families, we carried out a hierarchical multivariate analysis of variance (nested MANOVA) [two factors: full-sib family (2 families) nested in half-sib family (3 families)] for the number of tooth rows, the percentage of unicuspid teeth in the outer tooth row of the upper jaw, and the first three PCs of morphometric distances. We then performed nested ANOVAs on individual traits to investigate further which traits were significantly different among the three half-sib families and the six full-sib families.

RESULTS

Analysis of phenotypic variation and divergence

The number of tooth rows and percentage of unicuspid teeth in the outer tooth row varied greatly within and among islands (Fig. 2). The distribution of the number of tooth rows showed discontinuities and deviations from unimodality in the ecotypes of Makobe Island, but not at the other two locations. The distributions of percentage of unicuspid teeth showed two modes when ecotypes within each island were pooled together. At Bihiru these two modes had similar densities, whereas the nearly entirely bicuspid ecotype was clearly the more abundant at Makobe and Igombe. Principal components analysis identified three major axes of morphological variation (axes that explain more than 10% of the variation) (see Appendix Table S1). Scores of all three PCs were unimodally distributed in the three species (Fig. 2).

The nested ANOVAs revealed that the number of tooth rows, percentage of unicuspid teeth, and morphometric PC1 were highly significantly different between the ecotypes at some or at all islands (Table 2; see Appendix Fig. S2). *Neochromis* sp. 'unicuspid scraper' at Makobe Island had a smaller number of tooth rows, a higher percentage of unicuspid teeth, and lower PC1 values than *N. omnicaeruleus* (Fig. 3a, d). The number of tooth rows and morphological PC1 were also significantly differentiated in the same direction between *N. greenwoodi* and *Neochromis* sp. 'unicuspid scraper' (at Igombe island) (Fig. 3b, e; see Appendix Fig. S2). At Bihiru Island, on the other hand, the two putative ecotypes of *Neochromis* sp. 'Bihiru scraper' were significantly differentiated only in the percentage of unicuspid teeth (Fig. 3c, f; see Appendix Fig. S2).

The Pearson correlations revealed that at Makobe Island all three traits were highly inter-correlated ($P < 0.001$). At Igombe Island, the number of tooth rows and PC1 ($P = 0.01$) and percentage of unicuspid teeth and PC1 ($P = 0.031$) were also correlated, although these correlations did not remain significant after FDR correction. No significant correlations were found in the species of Bihiru Island.

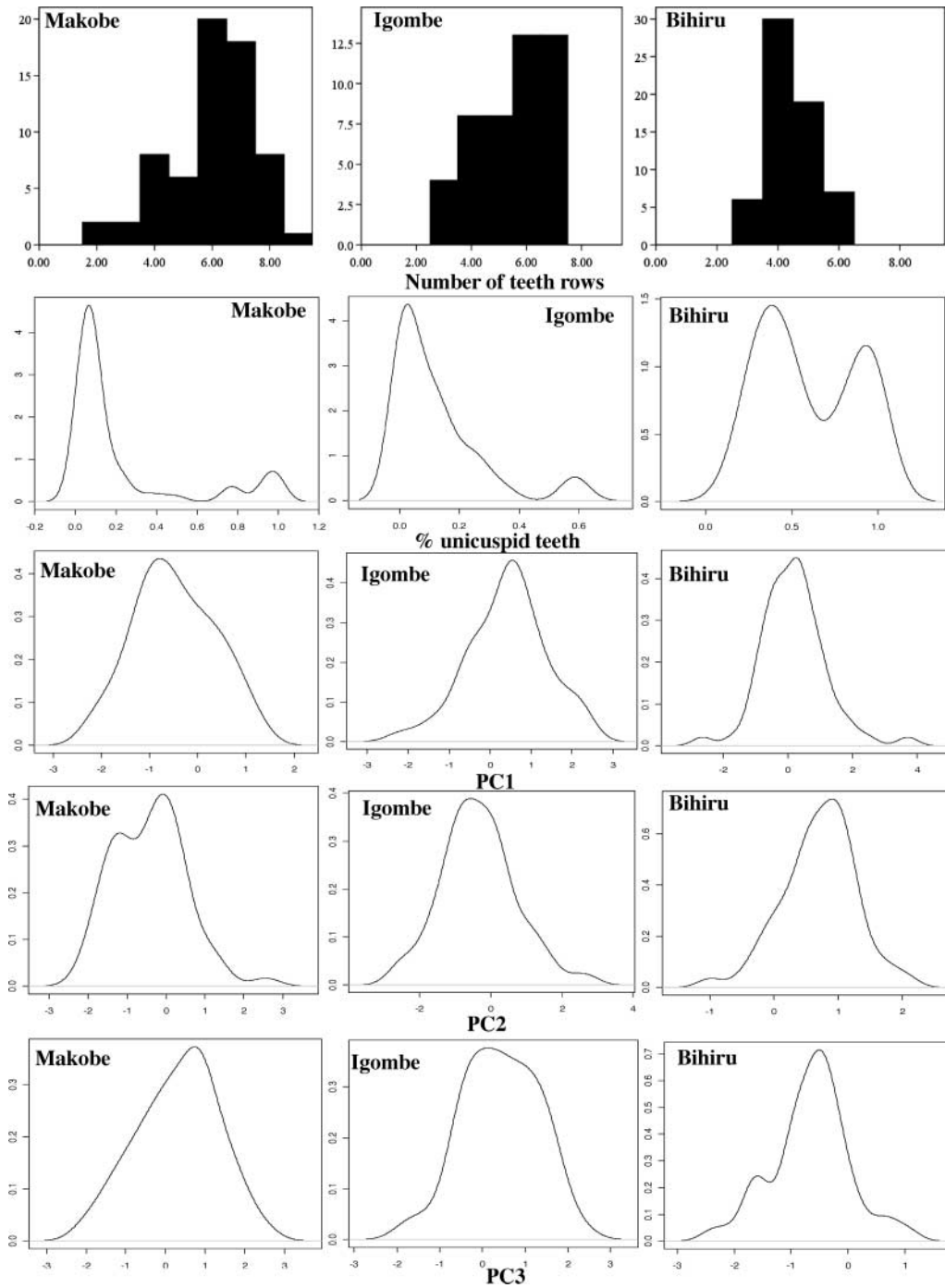


Fig. 2. Histograms for number of tooth rows and density plots (from top to bottom) for percentage of unicuspid teeth and PC1, PC2, and PC3 of morphological variation for populations from the three islands separately.

Table 2. Results of nested ANOVAs for number of tooth rows, percentage of unicuspid teeth, and the first three PCs of morphological variation for morphs nested within islands

Character	Ecotypes (island)			Island		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Percent unicuspid teeth	3	26.416	<0.001	2	53.146	<0.001
PC1	3	24.395	<0.001	2	23.114	<0.001
PC2	3	2.579	0.055	2	30.229	<0.001
PC3	3	0.550	0.649	2	11.973	<0.001
	d.f.	Wald's χ^2	<i>P</i>	d.f.	Wald's χ^2	<i>P</i>
Tooth rows	3	16.145	0.001	2	5.996	0.050

Note: Analyses of variance with a linear distribution were used for all variables except for number of tooth rows where a Poisson distribution was used. Significant *P*-values are in **bold**.

Analysis of neutral genetic variation and divergence

Most microsatellite markers exhibited high polymorphism (see Appendix Table S2). A total of 287 alleles were found over the nine loci, ranging from 8 to 72 per locus. No deviations from Hardy-Weinberg equilibrium were found in any of the ecotypes, except for *Neochromis* sp. 'unicuspid scraper', where deviations were observed for locus OSU20d. Significant linkage disequilibrium was found in ecotypes from Makobe and Igombe, but not from Bihiru (see Appendix Table S3).

The Neighbour-Joining tree based on F_{ST} values confirmed that ecotypes from the same island tended to be genetically most related (Fig. 4).

The population assignment test performed with STRUCTURE v.2.2 (Pritchard *et al.*, 2000) failed to recognize genetic structure between the six sampled units (ecotypes * islands) ($K=1$; mean estimated $-\ln$ probability of data = -8917.41 ; posterior probability = 0.993). This is likely a consequence of weak population structure and the low number of markers and individuals (Evanno *et al.*, 2005).

GENECLASS identified 12 individuals as first-generation migrants ($P < 0.01$). The number of migrants in each ecotype ranged from one (*N. omnicaeruleus*, *N. greenwoodi*, and *Neochromis* sp. 'Bihiru scraper' bicuspid) to four (*Neochromis* sp. 'Bihiru scraper' unicuspid). All migrants had the highest probability of being from sampled populations (see Appendix Table S4).

Neutral genetic variation overlapped largely among ecotypes within each island (Fig. 3g, h, i). The F_{ST} value between ecotypes from the same island was low ($F_{ST} = 0.0176$) but significant ($P < 0.001$) at Igombe. The F_{ST} value was even lower and non-significant at Makobe ($F_{ST} = 0.0032$). However, after grouping individuals as either having more than 50% unicuspid teeth ($n = 10$) or less than 50% unicuspid teeth ($n = 54$), the F_{ST} value became higher and significant ($F_{ST} = 0.009$, $P = 0.036$). The F_{ST} value at Bihiru was the lowest of all and not significant ($F_{ST} = 0.0027$), and this did not change after grouping populations by percentage of unicuspid teeth (more than 50% unicuspid teeth, $n = 32$; less than 50% unicuspid teeth, $n = 29$; $F_{ST} = 0.001$, $P = 0.360$). For Igombe, a comparison of tooth

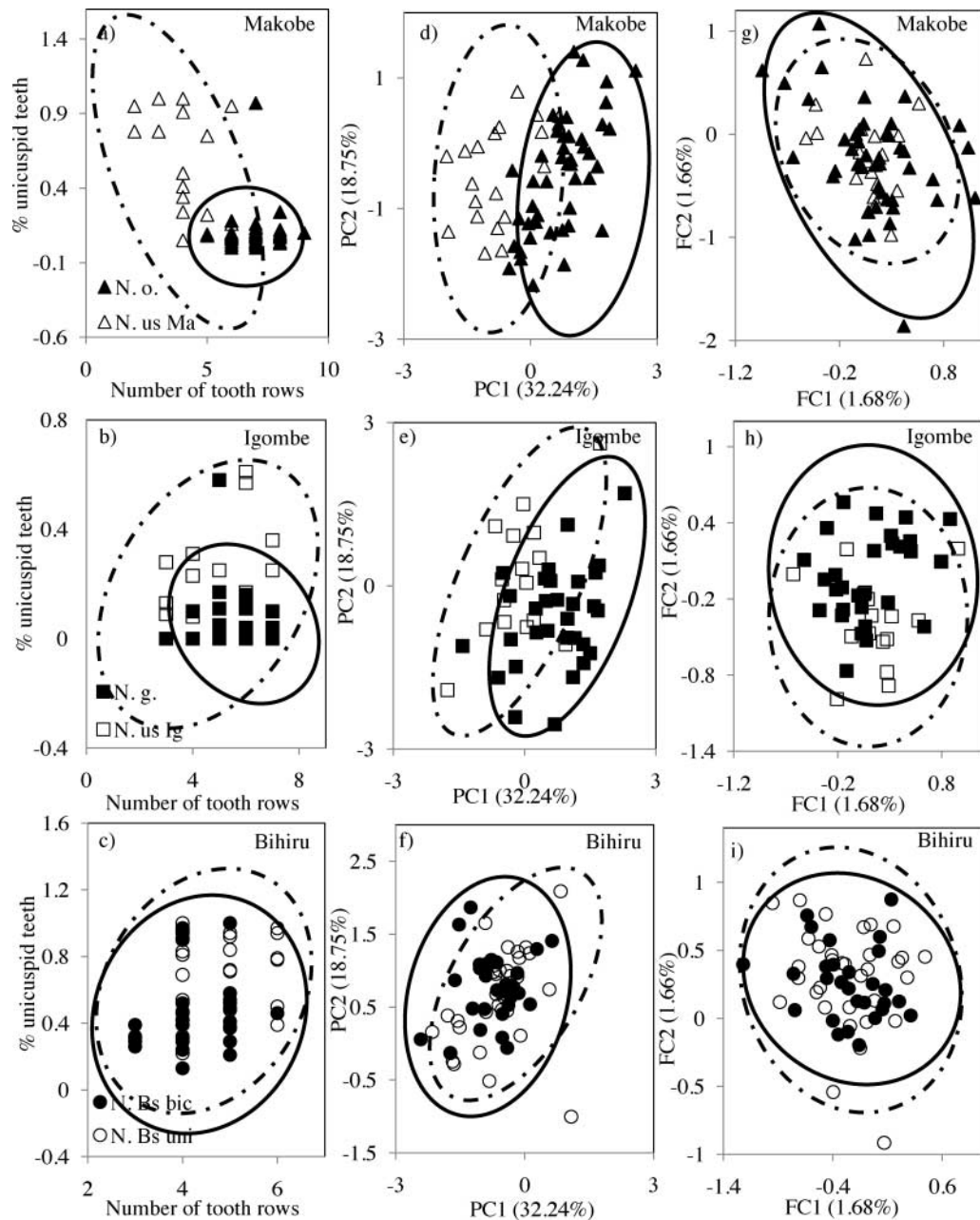


Fig. 3. Bivariate plots of number of tooth rows and percentage of unicuspid teeth (panels on the left: a, b, c), PC1 and PC2 of morphometry (panels in the middle: d, e, f), and FC1 and FC2 of neutral genetic markers (panels on the right: g, h, i). Ellipses represent 95% confidence limits.

shape groups could not be made because only three individuals had more than 50% unicuspid teeth. Single-locus F_{ST} values were generally low and had narrow distributions (Table 3).

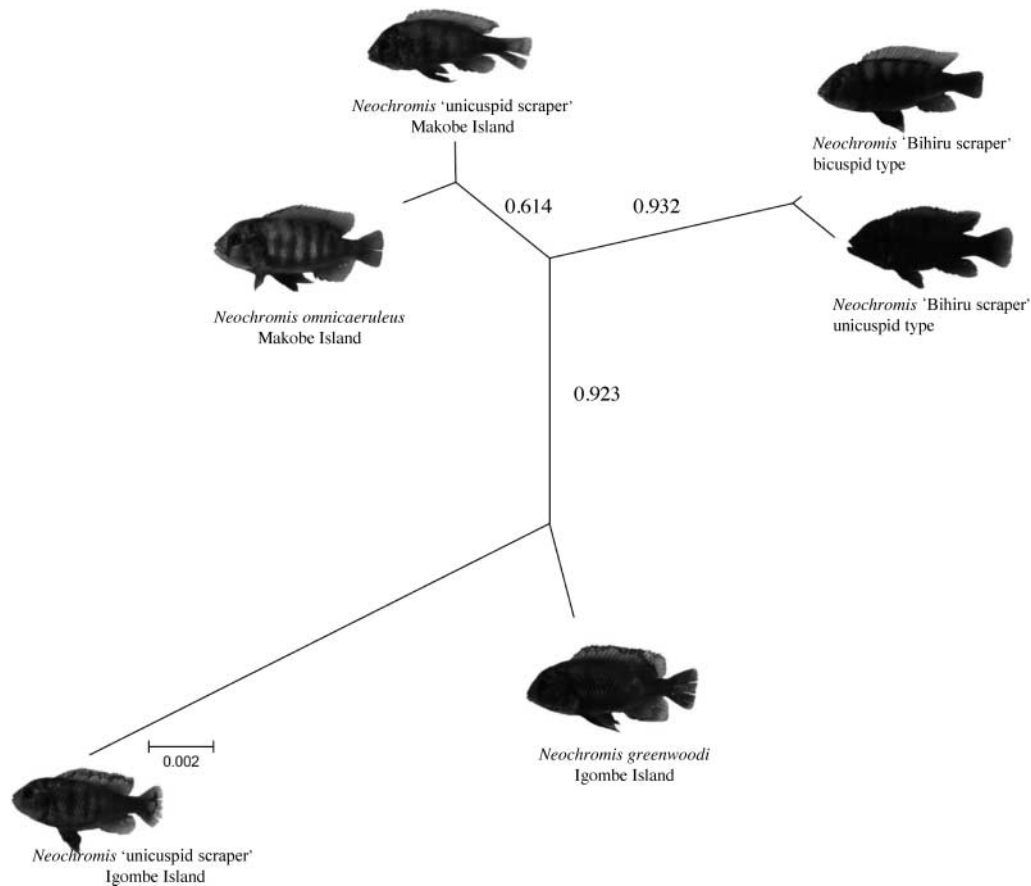


Fig. 4. Unrooted Neighbour-Joining tree constructed from F_{ST} values between ecotypes. The numbers next to the branches are bootstrap values for 1000 bootstrap resamplings of the nine loci typed for groups with more than 50% bootstrap support.

Phenotypic and genetic structure over water depth

The Mann-Whitney tests rejected the null hypothesis of equal depth distribution among ecotypes only at Makobe ($P < 0.001$): *N. omnicaeruleus* were caught at shallower depth than individuals identified as *Neochromis* sp. 'unicuspid scraper'. Also at Makobe, morphological PC1 and the number of tooth rows were significantly negatively correlated with water depth, and the percentage of unicuspid teeth was significantly positively correlated with water depth (Fig. 5). At Igombe Island, the distribution of number of tooth rows was significantly negatively associated with water depth. At Bihiru, the distribution of eco-morphological traits was not significantly associated with water depth. All significant relationships were in the direction predicted by the combination of the resource distribution and the functional morphological considerations, i.e. morphologies that suit algae-scraping feeding modes (many tooth rows, predominantly bicuspid teeth in the outer rows, high PC1 score – wider lower jaw, greater head width, deeper body) dominate in shallower water.

Table 3. Single-locus and multi-locus F_{ST} values between (a) ecotypes based on external morphology and (b) ecotypes based on quantification of dentition

Comparison	External morphology			Dentition	
	N.o.-N.us Ma	N.g.-N.us Ig	N.Bs bic-N.Bs uni	N.o.-N.us Ma	N.Bs bic-N.Bs uni
Ppun5	-0.01	0.027**	-0.004	0.000	0.008
Ppun7	0.006	0.011	0.025***	0.018	0.001
Ppun17	0.012	0.015	0.008	0.019	0.000
Ppun21	0.016	0.003	-0.004	0.019	0.000
Ppun32	-0.012	0.022	-0.015	0.001	0.000
OSU16d	0.007	0.022*	0.000	0.000	0.002
OSU19T	0.009	0.008	-0.006	0.016	0.003
OSU20d	0.013***	0.001	0.001	0.017*	0.000
TmoM5	-0.003	0.055***	0.011*	0.011*	0.007
Multi-locus	0.003	0.0193***	0.003	0.010*	0.001

Note: *Neochromis omnicaeruleus*, *N. greenwoodi*, *Neochromis* sp. ‘unicuspid scraper’ from Makobe and Igombe, and *Neochromis* sp. ‘Bihiru scraper’ bicuspid morph and unicuspid morph are represented by N.o., N.g., N.us Ma, N.us Ig, N.Bs bic, and N.Bs uni, respectively. Significant F_{ST} values are in **bold**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

At Igombe Island, water depth at capture was significantly related to neutral genetic structure: pairwise genetic distances among individuals were significantly positively correlated with the depth differential between them ($r = 0.1744$, $P = 0.015$). At Makobe, the same effect was close to significant ($r = 0.065$, $P = 0.053$), whereas no trend was observed at Bihiru ($r = 0.0012$, $P = 0.491$) (see Appendix Fig. S3).

Common garden experiment with *Neochromis* sp. ‘Bihiru scraper’ ecotypes

We found significant variation between unrelated half-sib families of *Neochromis* sp. ‘Bihiru scraper’ (hierarchical MANOVA: $F = 2.877$, d.f. = 10, $P = 0.005$) as well as between the two full-sib families nested within each of the half-sib families (hierarchical MANOVA: $F = 2.101$, d.f. = 15, $P = 0.016$). Subsequent nested ANOVAs on individual traits revealed that the three unrelated half-sib families differed significantly in the number of tooth rows and in PC1. There were significant differences in the percentage of unicuspid teeth between full-sib families (Table 4; see Appendix Fig. S4).

DISCUSSION

We investigated populations of cichlid fish in Lake Victoria that inhabit three locations varying in habitat heterogeneity and the steepness of habitat gradients and which have evolved replicate pairs of ecotypes. The extent of phenotypic and genetic differentiation of ecotypes varies among islands, reminiscent of variable stages of ecological speciation. We examined factors that may explain this variation.

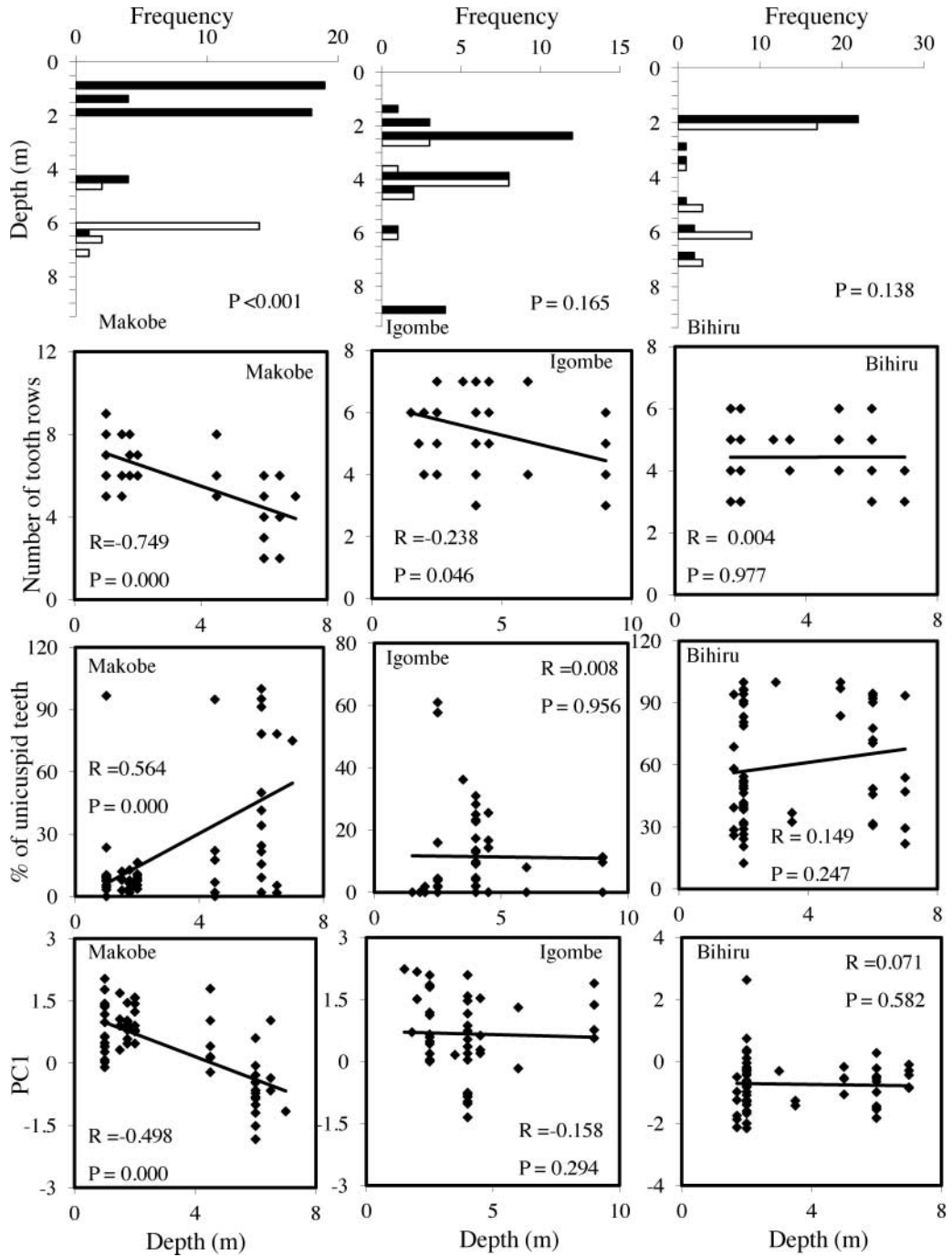


Fig. 5. Depth distribution (m) of individuals by (a) ecotype, (b) number of tooth rows, (c) percentage of unicuspid teeth, and (d) morphometric PC1 scores. Correlation coefficient (R) between depth and each variable and its significance (P -value) are also shown.

Table 4. Results of nested ANOVAs on the number of tooth rows, percentage of unicuspid teeth, and the first three PCs for males of three unrelated half-sib families and two full-sib families nested in each

Character	Half-sib families			Unrelated families		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Tooth rows	3	1.818	0.162	2	9.482	0.001
Percent unicuspid teeth	3	6.455	0.001	2	2.055	0.143
PC1	3	1.865	0.154	2	3.816	0.032
PC2	3	1.435	0.249	2	0.759	0.476
PC3	3	2.666	0.063	2	2.230	0.123

Note: Unrelated half-sib families do not share both mothers and fathers. Each of the three unrelated half-sib families is composed of two full-sib families that share the same mother but have different fathers. Significant *P*-values are in **bold**.

Stages of evolutionary divergence

Theory suggests competition for a limiting resource can lead to the evolution of disparity in functional traits and possibly even result in speciation (Rosenzweig, 1978; Doebeli and Dieckmann, 2003; Gavrilets, 2004; Bolnick and Fitzpatrick, 2007). However, gene flow between diverging populations may cancel this effect. We found the strongest eco-morphological differentiation at Makobe Island, where there were significant differences in tooth shape, the number of tooth rows, and several external morphological traits summarized in PC1. Differentiation at neutral marker loci was weak but significant. At Igombe Island, the ecotypes were differentiated by fewer morphological traits (number of tooth rows and morphology PC1) but neutral marker differentiation was somewhat higher. At both islands, we found significant correlations between trait values in several different morphological traits. The high frequency of significant linkage disequilibrium between multiple microsatellite loci, particularly when all genotypes were pooled, suggests that more than one population is present in our samples from Makobe and Igombe Islands. At Bihiru Island, on the other hand, the ecotypes differed only in tooth cusp shape. Based on the microsatellite F_{ST} value and the low amount of linkage disequilibrium, the ecotypes of *Neochromis* sp. 'Bihiru scraper' appear to belong to a single panmictic population, not inconsistent with the morphological data, for which no correlation between morphological traits was observed.

We expected phenotypic differentiation and neutral genetic differentiation to be correlated (Schluter, 2000; Hendry, 2009). Consistent with this expectation, the ecotype pair that exhibited the weakest eco-morphological differentiation displayed no significant neutral genetic differentiation. In contrast, the ecotype pair with the greatest eco-morphological differentiation did not also have the strongest neutral genetic differentiation. A lack of differentiation at neutral loci, however, should be interpreted with caution, as it can result from the low power of this inferential approach (Thibert-Plante and Hendry, 2010). Thus it is possible that the effects of ecologically driven reproductive barriers are simply yet to be detected at unlinked neutral loci, in which case progress towards speciation could be more advanced than our results suggest. Nonetheless, our results add to the growing number of studies showing that natural replicate pairs of sympatric or parapatric ecotypes that vary in their

extent of phenotypic differentiation also vary in differentiation at neutral markers of gene flow and arguably have reached different stages in the process of speciation (Seehausen *et al.*, 2008; Berner *et al.*, 2009; Nosil *et al.*, 2009; Renaut *et al.*, 2011; Kaeuffer *et al.*, 2012).

Phenotypic and genetic variation over water depth

Rock-dwelling cichlid fishes tend to be restricted to patches of rocky habitat, often separated from other such patches by long stretches of sandy or muddy lake floor. Our analysis revealed that ecotypes from the same island tended to be most genetically similar (Fig. 4), which could be the result of *in situ* divergence, secondary gene flow or both. Also, a few first-generation migrants were detected in each ecotype, but most individuals were assigned to the ecotype they belonged to, suggesting that ecological and short-term eco-evolutionary dynamics occur at the local (island) scale. Our results are consistent with the hypothesis of ecotypic divergence along the habitat gradient mediated by water depth. The dimensionality and strength of ecotypic divergence varied among islands, however. While phenotypic differentiation between depth habitats was strong and involved multiple dimensions of phenotype at Makobe Island, it was weaker and affected only one trait at Igombe Island, and even weaker at Bihiru Island where no trait was significantly associated with water depth. Although varying in strength and significance of correlations, all trends of eco-morphological differentiation along the water depth gradients were in the direction predicted by our hypothesis of adaptation in response to divergent selection on feeding resources along the habitat gradient: more bicuspid teeth, a higher number of tooth rows, and higher PC1 scores in shallow water. All of these traits are known to be functionally associated with feeding on firmly attached algae (Barel, 1983). Bicuspid tooth shape and a large number of tooth rows are direct adaptations to a feeding regime that relies on scraping attached algae from hard substrates (Witte and Van Oijen, 1990). *Neochromis omnicaeruleus* and *N. greenwoodi* are typical representatives of this trophic group. On the other hand, blue and green algae were found in the few individuals of *Neochromis* sp. 'unicuspid scraper' that had their stomach contents analysed (Seehausen, 1996). Therefore, it is probable that their diet consists of algae such as diatoms, not firmly attached to substrate, the removal of which does not require closely spaced tooth rows and cusps. Among the traits loading highly on PC1, lower jaw width increases the surface area of the dental brush and a wide head is needed to accommodate voluminous jaw muscles, both adaptations associated with algae scraping. It is thus reasonable to infer that the different feeding resources associated with habitat depth place different functional demands on the eco-morphology of benthic-feeding cichlids and affect the depth distribution of ecomorphs.

In the present study, the habitat gradients were roughly between 20 and 300 m from the surface to the deep end of the rocky habitat (Seehausen and Bouton, 1997). Individual dispersal distances of *Neochromis* are not known, but non-territorial fish can occasionally be observed cruising distances of 10 m or more in a matter of minutes. Hence, the spatial extent of our gradients permits relatively high amounts of dispersal along the depth gradients, and also broad overlap of ecotypes' depth distribution at all islands (Fig. 4). Indeed, as a SCUBA diver, one regularly encounters territorial males of both ecotypes within a radius of a few metres. This provides an opportunity for negative frequency-dependent selection arising from within-morph competition for resources to facilitate local coexistence of the ecotypes, a process suggested by some modelling studies to be required for completion of speciation on gradients (Doebeli and Dieckmann, 2003; Gavrillets, 2004; Kawata *et al.*, 2007).

A relatively shallow and spatially linear gradient of light-dependent resources generated by a gently sloping lake floor and small rock boulders that create homogeneous exposure of substrate to sunlight, such as at Makobe Island, seems to be conducive to eco-morphological differentiation of ecotypes over water depth. On the other hand, a gradient incorporating a steep lake floor and very large rock boulders, generating an environment with only small patches of sunlight-rich primary-production-dominated shallow water, such as at Bihiru Island, appears to be too steep and irregular to allow for a strong correlation between habitat and eco-morphology to evolve.

Similarly, gene flow appears to be restricted between habitat depth-associated ecotypes at Makobe and Igombe but not at Bihiru Island, where the habitat gradient is irregular and slopes steeply. Our data thus appear consistent with the idea that coincidence of dietary (alpha) and habitat (beta) niche divergence facilitates speciation, even though a sample size of three replicates of ecotypic divergence does not permit any strong conclusions.

Proximate causes of phenotypic differentiation

We found significant, albeit low dimensional, phenotypic differentiation among ecotypes even where there was no indication of gene flow restrictions (i.e. at Bihiru Island). Such phenotypic differentiation could be due to phenotypic plasticity (West-Eberhard, 2003). If the ecotype differentiation were entirely due to phenotypic plasticity, we would expect no morphological differences between families of *Neochromis* sp. 'Bihiru scraper' raised in a common environment. Instead, we found significant differences in the percentage of unicuspid teeth among full-sib families within each half-sib family, and significant differences in the number of tooth rows and in morphometric PC1 among unrelated half-sib families. This suggests that the eco-morphological differentiation in the wild is not just a plastic phenotypic response to local variation in the resources. A larger number of families and formal assessment of heritability for different traits are needed to further analyse the genetics underlying trait differences among the ecotypes. Nonetheless, our results indicate heritable variation in eco-morphological traits within a single polymorphic population. Studies on crosses of unrelated cichlid species with different dental traits have found that marked differences in cichlid fish tooth cusp shape can be controlled by a single gene with major effect (Albertson *et al.*, 2003; Streelman and Albertson, 2006). If applicable to *Neochromis*, this would explain how considerably different tooth shapes can be maintained between ecotypes even in a panmictic population. Heritability of skull and jaw shape has been shown to be high for Lake Malawi cichlids, with a small number of genes with additive effects controlling individual skeletal elements (Albertson *et al.*, 2003). Studies on morphological differences between Lake Victoria cichlid species also support the idea that major components of morphological differentiation between species are heritable, although they also provide evidence for adaptive phenotypic plasticity in several morphological traits (Bouton *et al.*, 1999, 2002; Magalhaes *et al.*, 2009). Further experimental quantitative genetic studies are needed to characterize the genetic architecture and estimate heritability of morphology and dentition.

CONCLUSIONS

The three replicate cases of the same kind of ecotypic polymorphism analysed here are associated with phenotypic adaptation to gradients of depth habitat but vary in their extent of phenotypic differentiation and reproductive isolation, reminiscent of different stages of

ecological speciation. Differences between the three island environments may be at least partly responsible for the variation in the extent of ecological and genetic differentiation observed. Together our results are consistent with the notion that the interaction between divergent selection and gene flow, mediated by spatial environmental structure, determines variation in the process of speciation.

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