Evolution of body shape in differently coloured sympatric congeners and allopatric populations of Lake Malawi's rock-dwelling cichlids

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Abstract

The cichlid fishes of Lake Malawi represent one of the most diverse adaptive radiations of vertebrates known. Among the rock-dwelling cichlids (mbuna), closely related sympatric congeners possess similar trophic morphologies (i.e. cranial and jaw structures), defend overlapping or adjacent territories, but can be easily distinguished based on male nuptial coloration. The apparent morphological similarity of congeners, however, leads to an ecological conundrum: theory predicts that ecological competition should lead to competitive exclusion. Hence, we hypothesized that slight, yet significant, ecological differences accompanied the divergence in sexual signals and that the divergence of ecological and sexual traits is correlated. To evaluate this hypothesis, we quantified body shape, a trait of known ecological importance, in populations of *Maylandia zebra*, a barred, widespread mbuna, and several sympatric nonbarred congeners. We found that the barred populations differ in body shape from their nonbarred sympatric congeners and that the direction of shape differences was consistent across all barred vs. nonbarred comparisons. Barred populations are generally deeper bodied which may be an adaptation to the structurally complex habitat they prefer, whereas the nonbarred species have a more fusiform body shape, which may be adaptive in their more open microhabitat. Furthermore, *M. zebra* populations sympatric with nonbarred congeners differ from populations where the nonbarred phenotype is absent and occupy less morphospace, indicating potential ecological character displacement. Mitochondrial DNA as well as published AFLP data indicated that the nonbarred populations are not monophyletic and therefore may have evolved multiple times independently. Overall our data suggest that the evolution of coloration and body shape may be coupled as a result of correlational selection. We hypothesize that correlated evolution of sexually selected and ecological traits may have contributed to rapid speciation as well as the maintenance of diversity in one of the most diverse adaptive radiations known.

Introduction

Adaptive radiations are characterized by rapid ecological divergence of lineages and simultaneous bursts of speciation (Schluter, 2000). In vertebrates, it has been

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hypothesized that three stages of adaptive radiations can be distinguished (Streelman & Danley, 2003). The first stage, characterized by divergence in macrohabitat use, and the second stage, identified by trophic niche differentiation, are associated with divergence at higher taxonomic levels (i.e. macrohabitat clades and genera, respectively). The diversification of species within genera is thought to have occurred during the third stage of the diversification and resulted in morphologically similar congeners differing in characters used for sexual

communication (Sturmbauer, 1998; Danley & Kocher, 2001; Salzburger, 2009). The differentiation solely in sexual signals among sympatric congeneric species during the third stage, however, leads to an ecological conundrum, as theory predicts that ecological competition should lead to competitive exclusion (Koplin & Hoffmann, 1968; Armstrong & McGehee, 1980; Kaplan & Denno, 2007; Anderson, 2008). Hence, the stable coexistence of species differing in sexual signalling should be accompanied by ecological trait divergence that mitigates interspecific competition among closely related species. Indeed, it has been shown that diversification in sexual signals can be associated with divergence in ecologically relevant traits in some adaptive radiations (Price, 1998; Boughman, 2001; Podos, 2001; Streelman & Danley, 2003; Servedio et al., 2011; Derryberry et al., 2012). Yet, such associations have received little attention in some of the most diverse vertebrate radiations, such as parrotfish and cichlids (see Streelman & Danley, 2003).

The haplochromine cichlids from East Africa are a model system for the study of adaptive radiation due to their young age and extreme phenotypic, ecological, and behavioural diversity (Seehausen, 2006; Salzburger, 2009; Sturmbauer et al., 2011). With more than 2000 species in the three East African Great Lakes (Tanganyika, Victoria and Malawi), cichlids represent the most species-rich vertebrate radiations known (Genner et al., 2004; Koblmüller et al., 2008). Lake Malawi, with more than 700 endemic species of haplochromine cichlids, supports the most species-rich species flock of East African cichlids (Kocher, 2004; Danley et al., 2012). Local cichlid communities can include several dozens of closely related taxa (Ribbink et al., 1983; Ding et al., in review), with as many as five different congeneric species coexisting at a single location (Ribbink et al., 1983; Genner & Turner, 2005; Ding et al., in review). This poses several interrelated questions: (i) Do sympatric congeners differing primarily in sexual signalling traits vary in ecologically relevant traits? (ii) Is ecological trait differentiation consistent across replicated colour phenotypes? and (iii) Have similar colour phenotypes in different localities evolved independently or do they have a common origin?

To investigate the ecological differentiation of sympatric congeners, we studied the genus *Maylandia*, the most species-rich mbuna genus in Lake Malawi. Species of this genus share a similar body shape and trophic morphology, but differ in their male nuptial coloration (Stauffer et al., 1997, 2013; Allender et al., 2003). Coloration in cichlids is an important sexual signal as species with different nuptial coloration generally appear to be reproductively isolated (Couldridge & Alexander, 2002; Kidd et al., 2006), and the experimental disruption of male coloration can lead to the breakdown of species barriers (Seehausen & van Alphen, 1998). Our study examined *M. zebra*, a blue bodied species with distinctive black vertical bars along the body (Figs 1 and 2). *Maylandia zebra* is the most widespread *Maylandia* species and can be found at nearly all rocky habitats in the lake. In contrast, the majority of *Maylandia* species are endemic to a limited number of rocky outcrops within the lake where they are almost always sympatric with *M. zebra* (Ribbink et al., 1983). Several of these species are blue and lack the eponymous bars of *M. zebra* (Figs 1 and 2). Behavioural studies of a sympatric species pair suggested that, although the barred and nonbarred species tend to occupy adjacent territories, their microhabitat preferences might differ (Danley, 2011), and visual cues, such as the presence or absence of body bars, are sufficient to elicit conspecific mate recognition (Kidd et al., 2006).

The existence of multiple *M. zebra* populations with and without a sympatric, nonbarred congener offers the opportunity to test the role of fine scale ecological trait differentiation in mediating coexistence of closely related species. Previous research identified geographically distinct populations of *M. zebra* that differ in body size and tropic morphology (Streelman et al., 2007) but could not identify the role that sympatric congeners might play in generating this variation. Furthermore, sympatric congeners exploit similar food sources (Martin & Genner, 2009), but can show slight differences in microhabitat use, territory size and territory defence (Holzberg, 1978; Danley, 2011). Studies in other systems have shown that even such slight differences can lessen competition and allow for coexistence (Willis et al., 2005). Nonetheless, it remains unclear whether differential habitat use is reflected in phenotypic trait divergence. Thus, we hypothesized that diversification in sexual signals is associated with divergence in ecologically relevant traits in this genus of Lake Malawi cichlids.

In fish, body shape is known to respond to a variety of ecological sources of selection including predation (Langerhans, 2009; Ingram et al., 2012), competition (Kassam et al., 2003; Kerschbaumer et al., 2014) and local abiotic conditions (Tobler & Carson, 2010; Tobler et al., 2011). In cichlids, body shape can be used to quantify even small differences in adaptive morphology (reviewed in Kerschbaumer & Sturmbauer, 2011). Consequently, we studied variation in body shape among communities of *Maylandia* in which barred and nonbarred species existed in sympatry (sympatric populations) and populations of *M. zebra* that lacked a sympatric nonbarred congener (nonsympatric populations) to test a series of hypotheses in regard to ecological differentiation between *Maylandia* species and among *M. zebra* populations. First, we hypothesized that sympatric congeners differing in the presence or absence of body bars also differ in body shape. Second, we tested the hypothesis that these differences are consistent across replicate species pairs found at different locations, that is, body shape in all nonbarred species differs from the barred species in a similar manner. Third, we
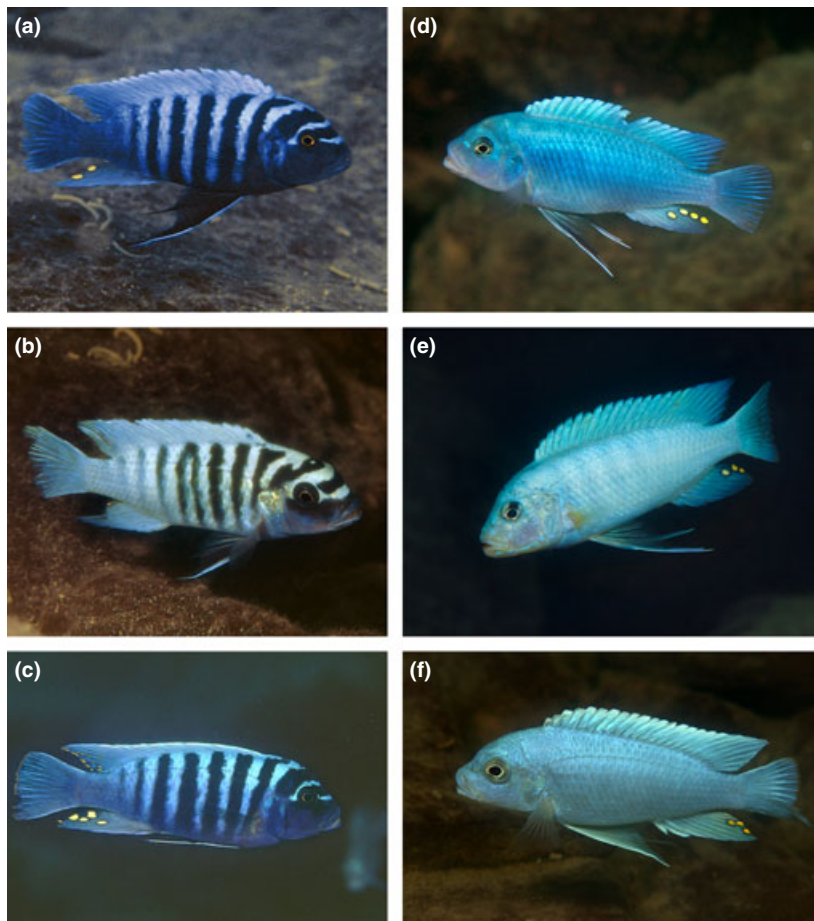
Fig. 1 *Maylandia zebra* from (a) Nkhata Bay, (b) Mazinzi Reef and (c) Chiofu Bay and (d) *Maylandia callainos* from Luwino Reef (close to Nkhata Bay), (e) *Maylandia benetos* from Mazinzi Reef and (f) *Maylandia chrysomallos* from Gome (close to Chiofu Bay).

used genetic markers and Bayes factor analysis to investigate whether potentially consistent differences between replicated species pairs are the result of common ancestry or convergent evolution. Specifically, we hypothesized that taxa with similar colour pattern are monophyletic. Fourth, we tested for morphological differences between populations of *M. zebra* that either were sympatric with a nonbarred congener or lacked a nonbarred congener. If ecological differentiation plays a role for the coexistence of species, we hypothesized that the body shape of sympatric *M. zebra* populations sympatric with a nonbarred species differs from those that lack a nonbarred congener and that populations lacking the congener are morphologically more variable. Evaluating this set of hypotheses then provides more general conclusions about potential mechanisms of species divergence and persistence in rapidly diverging systems.

Materials and methods

Specimen collection

Our study focused on species within the genus *Maylandia*. The appropriate genus name for this group is

contended, and the species studied here have been classified as belonging to the genus *Metriaclima* or, historically, *Pseudotropheus* (Regan, 1922; Stauffer *et al.*, 1997, 2013). Here, we follow the *Maylandia* designation (Meyer & Foerster, 1984; Condé & Géry, 1999) as this name has been widely adopted in Gen-Bank.

In 2010, we collected *M. zebra* at six locations (Nkhata Bay, Chiofu Bay, Mazinzi Reef, Boadzulu, Otter Point and Illala Gap, Table 1, Figs 1 and 2). At three of the locations, we collected a sympatric nonbarred species: *Maylandia benetos* (Mazinzi Reef), *Maylandia chrysomallos* (Chiofu Bay) and *Maylandia callainos* (Nkhata Bay). A total of 193 males were obtained. At some of the locations, additional species of *Maylandia* can be found which are morphologically more divergent from *M. zebra* and were therefore not included in this study. In this manuscript, we refer to *M. zebra* populations that are sympatric with a blue congener as 'sympatric' populations and to populations of *M. zebra* that lack a sympatric blue congener as 'nonsympatric' populations following the terminology of Kerschbaumer *et al.* (2014). The distance between sampling locations varied between < 2 km to more than 300 km (Fig. 2).
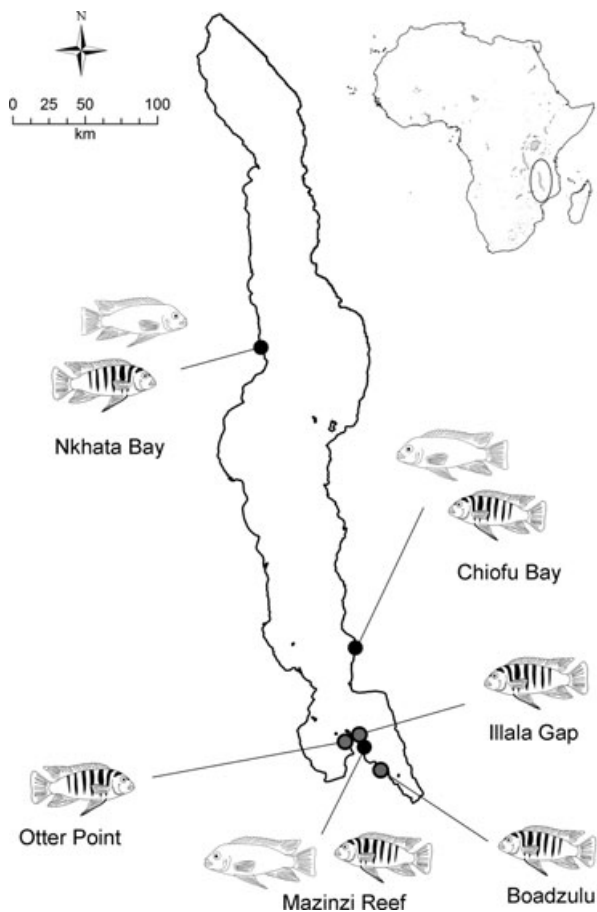
Fig. 2 Map displaying the sampling sites for 'sympatric' species pairs of *Maylandia* (black dots) and 'nonsympatric' populations of *Maylandia zebra* (grey dots).

Specimens were caught in gill nets while SCUBA diving and photographed using a Canon Eos 540d (Canon Deutschland GmbH, Krefeld, Germany). After pictures were taken, the standard length was measured and specimens were fin clipped and released back at the original collection site. As a result of past studies, which showed clear effects of sex and allometry on body shape (Herler *et al.*, 2010; Kerschbaumer & Sturmbauer, 2011), we focused our analyses on adult males.

Geometric morphometrics and statistical analyses

We quantified body shape variation in different *Maylandia* populations using geometric morphometric analyses (Adams *et al.*, 2004). Lateral pictures of individual fish were imported into TPSDIG v.2.16 (Rohlf, 2006). On each picture, 16 homologous landmarks were identified and marked (Fig. 3, see figure caption, for a description of the landmarks). To quantify the ecological differentiation within and between *Maylandia* species, we conducted our analyses on two separate datasets. The first

dataset included species pairs from locations at which *M. zebra* and nonbarred species were sympatric; the second dataset included all *M. zebra* populations (sympatric and nonsympatric). For each dataset, landmark coordinates were aligned using least-square superimposition as implemented in the program tpsRelw (Rohlf, 2007) to remove effects of translation, rotation and scale. Based on the aligned coordinates, we generated a weight matrix consisting of partial warp scores with uniform components for each individual. To reduce data dimensionality, we subjected the weight matrices to a principal component analysis (PCA) based on the covariance matrix of the morphometric data. Unless otherwise stated, all statistical analyses were performed using SPSS v. 20 (IBM Inc., Armonk, NY, USA).

With the data collected from *M. zebra* individuals and their sympatric nonbarred congeners, we wanted to address two questions: (i) Is there shared site-specific variation in the barred and nonbarred forms that could represent adaptation to the local environment? and (ii) Do barred and nonbarred forms consistently differ from each other, indicating predictable differentiation at each locality? To do so, individual PC axes scores were used as dependent variables in a multivariate analysis of covariance (MANCOVA). Assumptions of multivariate normal error and homogeneity of variances and covariances were met for all analyses performed. *F*-values were approximated using Wilks' lambda and effect strengths by use of partial eta squared (η_p^2). We included species (barred vs. nonbarred) and sampling site as independent variables and used the standard length as a covariate to control for multivariate allometry. To visualize variation between species and across sites, we calculated divergence scores for each individual based on species (barred vs. nonbarred) and site divergence vectors as defined by Langerhans (2009). Such divergence vectors summarize the linear combination of shape variables that contribute most to variation in body shape for any given term in a MANOVA, while simultaneously controlling for other effects and not distorting morphological space. Individual scores were then used as independent variables in tpsRegression (Rohlf, 2005) to generate thin-plate spline deformation grids highlighting shape differences between groups. Variation in divergence scores was further studied using a mixed-model nested analysis of covariance (ANCOVA) to scrutinize the results of the MANCOVA (Langerhans, 2009).

With the complete *M. zebra* data, we wanted to test the hypothesis that 'sympatric' and 'nonsympatric' *M. zebra* populations consistently differed in body shape. Again, individual PC axes scores were used as dependent variables in a multivariate analysis of covariance (MANCOVA). We included 'sympatric' vs. 'nonsympatric' and sampling site (nested within 'sympatric' vs. 'nonsympatric') as independent variables and used the standard length as a covariate to control for multivariate

Table 1 Sampling list indicating the sampling location, species identity, the coloration phenotype of the respective species, the numbers of samples used for morphometrics and genetic analyses, and the respective GenBank accession numbers.

Location	Species	Phenotype	Number of samples (morphometrics)	Number of sequences	GenBank accessions
Boadzulu Island	*Maylandia zebra*	Barred	21	14	KC208919–KC208932
Mazinzi Reef	*M. zebra*	Barred	26	26	KC208879–KC208904
Mazinzi Reef	*Maylandia benetos*	Nonbarred	22	29	KC208850–KC208878
Illala Gap	*M. zebra*	Barred	27	19	KC208974–KC208992
Otter Point	*M. zebra*	Barred	18	23	KC208951–KC208973
Chiofu Bay	*M. zebra*	Barred	25	14*	GU128640–GU128653
Chiofu Bay	*Maylandia chrysomallos*	Nonbarred	23	18	KC208933–KC208950
Nkhata Bay	*M. zebra*	Barred	21	15*	GU128829–GU128843
Nkhata Bay	*Maylandia callainos*	Nonbarred	10	14	KC208905–KC208918
Total			193	172	

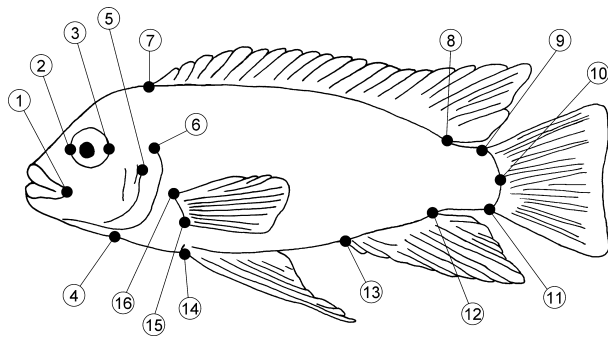*Sequences generated by Genner *et al.* (2010).

Fig. 3 The 16 landmarks analysed in this study: (1) most posterior point of the lips, (2) anterior edge of the eye, (3) posterior edge of the eye, (4) ventral tip of cleithrum, (5) dorsal end of pre-opercular groove, (6) dorsal origin of operculum, (7) anterior insertion of dorsal fin, (8) posterior insertion of dorsal fin, (9) upper insertion of caudal fin, (10) midpoint of the origin of caudal fin, (11) lower insertion of caudal fin, (12) posterior insertion of anal fin, (13) anterior insertion of anal fin, (14) anterior insertion of pelvic fin, (15) ventral insertion of pectoral fin, and (16) dorsal insertion of pelvic fin.

allometry. As with the previous dataset, we calculated divergence scores for each individual based on the 'sympatric' vs. 'nonsympatric' and site divergence vectors as defined by Langerhans (2009). These scores were used for visualization. However, as random nested factors are not applicable for MANCOVAs and the use of fixed effects can inflate type I error rates when nested terms are significant, we also investigated variation in divergence scores using ANCOVA to scrutinize the results of the MANCOVA (Langerhans, 2009).

To quantify the effects of geographical distance and gene flow on phenotypic divergence, we conducted partial Mantel tests. To wit, we calculated pairwise phenotypic distances by first removing the effects of size using a preparatory MANCOVA and used the resulting residuals to calculate pairwise Euclidean distances

between all multivariate population means (for details see Tobler & Carson, 2010). Pairwise phenotypic distances were then used as dependent variables for partial Mantel tests with 10 000 randomizations, as implemented in FSTAT (Goudet, 1995). Predictor matrices for the comparison between barred and nonbarred sympatric populations included colour type (same or different), geographical distance (log transformed in kilometres) and pairwise genetic distances (Φ_{st} values from the analyses described below). Predictor matrices for the comparison between 'sympatric' and 'nonsympatric' barred populations included coexistence type ('sympatric' or 'nonsympatric'), geographical distance (log transformed in kilometres) and pairwise genetic distances (Φ_{st} values from the analyses described below).

Molecular analyses

Fish were fin clipped in the field, and the tissue was either dried or stored in a preservative (20% DMSO, 0.25 M EDTA, saturated with NaCl, pH = 7.5) until further processing. DNA was isolated using the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol for tissue samples. We amplified a 442-bp fragment of the mitochondrial DLoop for 143 specimens using the forward primer HapThr-2 þ 4: 5′ CCTACTCCCAAAGCTAGGATC 3′ and the reverse primer Fish12s: 5′ TGCGGAGACTTG-CATGTGTAAG 3′ (Joyce *et al.*, 2005). PCR was performed using the following setup: 12.2 µL of diH$_2$O, 2 µL of 10× PCR buffer (reaction concentration 1×), 1.6 µL of dNTP mixture (0.2 µM each; Finnzymes, Vantaa, Finland), 0.2 µL of DyNAzyme™ DNA Polymerase (1.2 U, Finnzymes), 1 µL of each primer (0.5 µM, Integrated DNA Technologies, Coralville, IA, USA) and 2 µL of DNA template (either pure extract, 1 : 10, 1 : 50 dilution) for a total volume of 20 µL. Amplification conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 58 °C 1 min, and

72 °C for 2 min, with a final elongation step at 72 °C for 10 min. Ten microlitre of the PCR product was purified using 4 μL ExoSAP-IT enzyme mix (Affymetrix, Santa Clara, CA, USA). The purified products were sequenced by the Sequencing Facility at Yale University. All sequences were deposited in GenBank (accession numbers are given in Table 1).

Our own sequences and 29 additional sequences from two populations obtained from GenBank (Genner & Turner, 2012) were aligned using GENEIOUS v. 6.0.3 (Drummond *et al.*, 2011); base calls were visually inspected. General statistics of sequence variation were calculated with DNASP v.5.10 (Librado & Rozas, 2009). Estimates of genetic differentiation between populations were calculated as Φ_{st} with ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer, 2010) and were tested for significance using 100 permutations. A haplotype network was constructed using TCS v. 1.21 (Clement *et al.*, 2000) with a connection limit of 95%. Gaps were treated as fifth state. Relationships between populations were reconstructed using a Bayesian approach as implemented in *BEAST (Heled & Drummond, 2010) which is part of the BEAST package v.1.6.1 (Drummond & Rambaut, 2007). For the Bayesian analysis, populations were predefined according to location and phenotype. We used the GTR + I + G substitution model as determined by JMOD-ELTEST v.2.1.2 (Posada, 2008). The tree prior was defined as a Yule process. We ran the MCMC simulation for 100 million generations and discarded a burn-in of 10%. The results were checked for convergence in TRACER v. 1.5 (Rambaut & Drummond, 2009) and visualized with DENSITREE v. 2.01 (Bouckaert, 2010). A consensus tree was generated using TREEANNOTATOR v.1.6.1 discarding a burn-in of 10% (implemented in the BEAST package v.1.6.1).

We tested for the convergence or common ancestry of the coloration phenotypes using a Bayes factors comparison (Kass & Raftery, 1995; Marek & Bond, 2006). Bayes factors analysis differs from more traditional hypotheses testing methods in not offering criteria for the absolute rejection of a null hypotheses, instead this method allows for the evaluation of alternative hypotheses in comparison with the null hypothesis (Kass & Raftery, 1995). The Bayes factor approach has been employed in a variety of phylogenetic studies and represents a valuable tool when different hypotheses need to be compared (Genner & Turner, 2012; Husemann *et al.*, 2012). We constrained our phylogeny to the topology reflecting the hypothesis of common ancestry and ran *BEAST with these constrained clades. We defined the topology generated by an unconstrained run as the null hypothesis, with populations being predefined as units. The log files from these analyses were then used as input for TRACER, which compares the likelihood scores of the alternative hypothesis with the unconstrained tree and generates Bayes factors. Bayes factors above 10 are considered unsupportive, whereas

Bayes factors lower than 10 are considered supportive for the respective topology (Marek & Bond, 2006).

Results

Morphological analyses

The PCA contrasting barred and nonbarred sympatric congeners yielded eight axes describing a cumulative variance of 75% in body shape. MANCOVA analysis showed significant effects of sampling site and species as well as an interaction of both (Table 2). The ANCOVA on the species divergence scores yielded significant effects for species and a species by site interaction (Table 2). A clear pattern emerges when plotting the divergence vectors for species vs. site (Fig. 4): along the species divergence vector axis, congeners can clearly be distinguished, independent of their sampling site. The nonbarred species are more fusiform with a lower body depth relative to *M. zebra*. Distinct differences can also be seen in the shape of the head, the orientation of the mouth and the height of the caudal peduncle. In addition, populations with similar coloration can be distinguished along the sampling site axis. On this axis, body depth is the primary difference between members of different communities. For example, both *M. zebra* and *M. benetos* from Mazinzi Reef have the lowest body depth, whereas *M. zebra* and *M. chrysomallos* individuals from Chiofu Bay are the stoutest.

Next, we examined the effect of a nonbarred congener on *M. zebra* populations. The PCA on this data set resulted in nine axes explaining a cumulative variance of 80% in body shape. MANCOVA analysis yielded significant results (Table 3), for both the coexistence status

Table 2 Results of the multivariate analysis of covariance (MANCOVA) and the analysis of covariance (ANCOVA) on the species scores performed to test for body shape differences between colour phenotypes in sympatric species pairs of *Maylandia* (species score) and between sites (sites score). *F*-ratios were approximated using Wilks' lambda and effect sizes were estimated using Partial Eta squared (η_p^2).

| Effect	*F*	Hypothesis d.f.	Error d.f.	*P*-value	η_p^2
MANCOVA					
Intercept	5.075	8	113	<0.001	0.264
Standard length	4.893	8	113	<0.001	0.257
Species	28.595	8	113	<0.001	0.669
Site	8.094	16	226	<0.001	0.364
Species*Site	8.379	16	226	<0.001	0.372
ANCOVA species score					
Intercept	2.976	1	117.9	0.087	0.025
Standard length	2.335	1	120.0	0.129	0.019
Species	48.81	1	2.0	0.019	0.960
Site	1.317	2	2.1	0.427	0.559
Species*Site	4.561	2	120.0	0.012	0.071
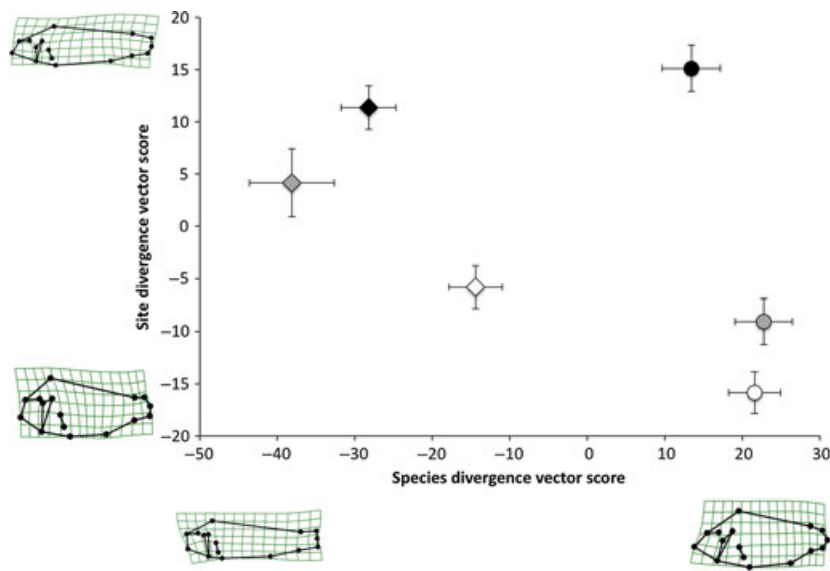
Fig. 4 Shape variation among species and sites. Colour-coding indicates different sites: Chiofu (white), Nkhata (grey), and Mazinzi (black). Symbols represent different phenotypes: barred (circles) and nonbarred (diamonds).

Table 3 Results of the multivariate analysis of covariance (MANCOVA) and the analysis of covariance (ANCOVA) on the species scores performed to examine body shape differences between 'sympatric' and 'nonsympatric' populations of *Maylandia zebra* and between sites. *F*-ratios were approximated using Wilks' lambda and effect sizes were estimated using Partial Eta squared (η_p^2).

Effect	*F*	Hypothesis d.f.	Error d.f.	*P*-value	η_p^2
MANCOVA					
Intercept	4.501	9.0	123.0	<0.001	0.248
Standard length	4.518	9.0	123.0	<0.001	0.248
'sympatric'/ 'nonsympatric'	13.300	9.0	123.0	<0.001	0.493
Site ('sympatric'/ 'nonsympatric')	10.332	36.0	462.7	<0.001	0.425
ANCOVA 'sympatric'/'nonsympatric' score					
Intercept	29.140	1.0	126.0	<0.001	0.188
Standard length	31.123	1.0	131.0	<0.001	0.192
'sympatric'/ 'nonsympatric'	10.995	1.0	4.2	0.027	0.723
Site ('sympatric'/ 'nonsympatric')	11.631	4.0	131.0	<0.001	0.262
ANCOVA site score					
Intercept	14.963	1.0	27.1	0.001	0.356
Standard length	23.835	1.0	131.0	<0.001	0.154
'sympatric'/ 'nonsympatric'	0.943	1.0	4.0	0.386	0.190
Site ('sympatric'/ 'nonsympatric')	83.740	4.0	131.0	<0.001	0.719

('sympatric'/'nonsympatric') of a population, as well as for the site effect (Table 3). The ANCOVA on the coexistence status divergence vector indicated that the presence/absence of a nonbarred congener and site had a significant influence on body shape, whereas the ANCOVA on the site divergence vector scores indicated that site

effects were significant, while the coexistence status ('sympatric'/'nonsympatric') was not (Table 3). The plot of morphological divergence along the site vector scores and the 'nonsympatric'/'sympatric' vector scores (Fig. 5) yielded some degree of differentiation of the 'nonsympatric' vs. 'sympatric' populations. Populations of *M. zebra* that are sympatric with a nonbarred congener generally have a deeper body, whereas populations without the congener can have rather low body depth. The head shape, however, appears rather similar independent of the presence or absence of the congener.

The results from the Mantel tests analysing the sympatric species pairs indicated that similarity in body shape is not correlated with genetic distance (Φ_{st}; $r = -0.049$, $P = 0.953$), geographical distance ($r = -0.198$, $P = 0.885$) and colour phenotype ($r = -0.363$, $P = 0.180$). Moreover, colour phenotype ($r = 0.073$, $P = 0.788$) and geographical distance ($r = 0.035$, $P = 0.991$) did not explain any variation in Φ_{st}. For the comparison of the six *M. zebra* populations, genetic distance significantly predicts morphological similarity (closely related populations are more similar, $r = 0.752$, $P = 0.013$). There is no evidence for effects of geographical distance ($r = 0.425$, $P = 0.252$) or of sympatry vs. allopatry ($r = 0.021$, $P = 0.945$) on body shape similarities.

Molecular analyses

We sequenced a 442-bp fragment of the mitochondrial DLoop for 143 specimens and obtained 29 additional sequences from the study by Genner and Turner (2012) for a total of 172 sequences belonging to nine populations of *Maylandia* (Table 1). The alignment contained five indels and 25 variable sites, 18 of which were parsimony informative. A total of 28 haplotypes were recovered. The total nucleotide diversity was 0.00604.

Fig. 5 Shape variation along the 'sympatric'/'nonsympatric' axis and among sites; black symbols indicate 'nonsympatric' populations of *Maylandia zebra*, whereas white symbols stand for populations of *M. zebra* being in sympatry with a nonbarred congener.
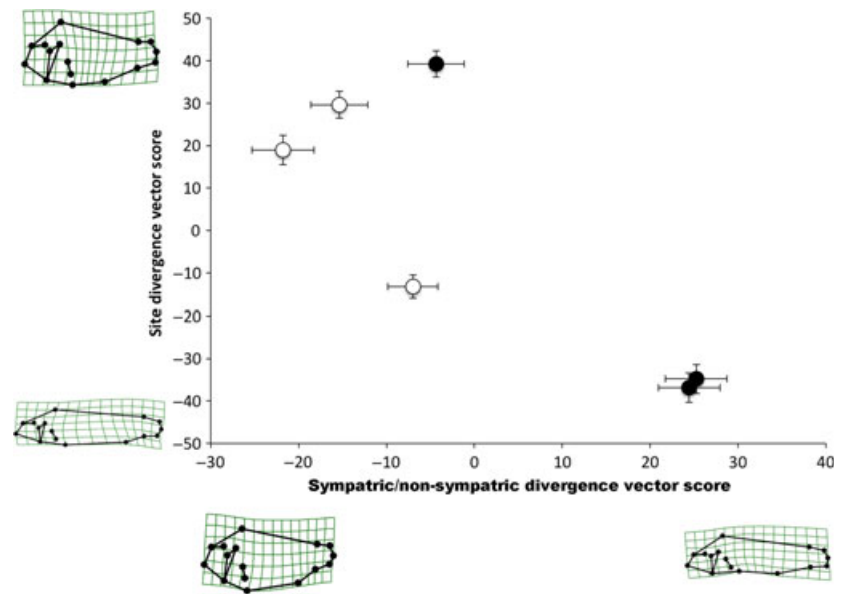
Φ_{st} estimates were generally high and ranged from 0.02573 (*M. zebra* from Boadzulu Island and *M. chrysomallos* from Chiofu Bay) to 0.93354 (between *M. benetos* from Mazinzi Reef and *M. zebra* from Otter Point). All comparisons except for the one, between *M. zebra* from Boadzulu Island and *M. chrysomallos* from Chiofu Bay, were significant (Table 4). Geographical distance did not explain the extent of genetic differentiation between populations ($Z = 815.8484$, $r = -0.1786$, one-sided $P = 0.7752$).

The haplotype network showed no clear structure (Fig. 6). All haplotypes were closely related, and no more than three mutational steps separated any haplotype. *Maylandia benetos* from Mazinzi Reef was the least diverse population with only a single haplotype, whereas *M. zebra* from Chiofu Bay was the most diverse with eight haplotypes. Across all species, three haplotypes were especially common. The first of these

common haplotypes was shared only between individuals from Illala Gap and Otter Point, whereas the second common haplotype was found in *M. benetos* and *M. zebra* from Mazinzi Reef and *M. callainos* from Nkhata Bay. The third common haplotype was most widely distributed and was found in *M. callainos* and *M. zebra* from Nkhata Bay, *M. chrysomallos* from Chiofu Bay and *M. zebra* from Boadzulu Island, Otter Point and Illala Gap. A fourth less common haplotype was shared between *M. callainos* from Nkhata Bay and *M. zebra* from Illala Gap. All other haplotypes were location specific.

Our efforts to reconstruct the relationships among populations and species yielded a tree with low support (Appendix S1). However, the tree shows one well-supported major split (pp = 100), which groups *M. benetos* and *M. zebra* from Mazinzi Reef together with *M. callainos* from Nkhata Bay and separates these three populations from all others. The only other well-

Table 4 Φ_{st} estimates of genetic differentiation generated from D-Loop sequences for all populations and species with Arlequin. Significance ($\alpha = 0.05$) as determined by 100 permutations is indicated by an asterisk; Mb, *Maylandia benetos*; Mz, *Maylandia zebra*; Mcal, *Maylandia callainos*; Mchr, *Maylandia chrysomallos*.

	Mb – Mazinzi Reef	Mz – Mazinzi Reef	Mcal – Nkhata Bay	Mz – Boadzulu Island	Mchr – Chiofu Bay	Mz – Chiofu Bay	Mz – Nkhata Bay	Mz – Otter Point
Mb – Mazinzi Reef	0							
Mz – Mazinzi Reef	0.58675*	0						
Mcal – Nkhata Bay	0.55642*	0.28386*	0					
Mz – Boadzulu Island	0.92396*	0.28779*	0.24423*	0				
Mchr – Chiofu Bay	0.85634*	0.28456*	0.24883*	0.02573	0			
Mz – Chiofu Bay	0.65488*	0.32338*	0.25353*	0.22935*	0.21434*	0		
Mz – Nkhata Bay	0.79713*	0.29979*	0.24814*	0.16691*	0.12738*	0.23688*	0	
Mz – Otter Point	0.93354*	0.80098*	0.76088*	0.86572*	0.84848*	0.70779*	0.81670*	0
Mz – Illala Gap	0.93191*	0.78976*	0.74743*	0.85690*	0.83952*	0.68648*	0.80377*	0.09106*
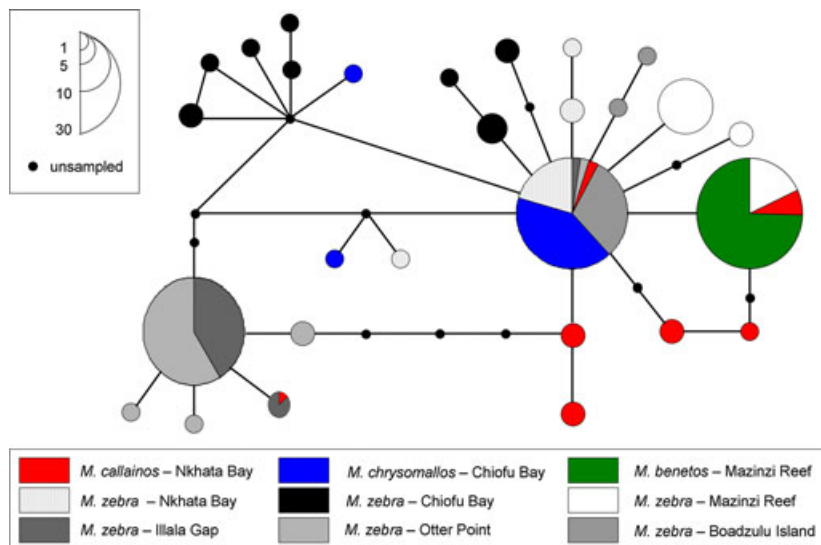
supported node (pp = 94) groups the *M. zebra* populations from Illala Gap and Otter Point together, which represent the two geographically closest locations. All other branches have very low support and the relationships cannot be regarded as reliable. In addition, we reanalysed AFLP data provided by Allender *et al.* (2003), which included *M. benetos* and *M. zebra* from Mazinzi Reef, and *M. callainos* from Nkhata Bay, as well as several other *Maylandia* species. We applied Bayesian and Neighbornet phylogenetic methods to the AFLP data. Neither approach provided good resolution, yet both indicated that the species with the nonbarred phenotype are not monophyletic and species with different coloration phenotypes group together (Appendix S2, also see Allender *et al.*, 2003 for more details). Our Bayes factor analysis clearly rejected monophyly of the coloration phenotypes (Bayes factor: 941.002; H_A: clustering by coloration phenotypes -883.347 ± 0.094 (mean LnL $\pm$ SE); unconstrained tree H_0: LnL -876.5 ± 0.118).

Discussion

Divergence of sympatric species pairs

Ecological theory predicts that stable coexistence is only possible if sympatric species have diverged in ecological and mating traits (Gause, 1934; Armstrong & McGehee, 1980; Dieckmann & Doebeli, 1999; Vandermeer *et al.*, 2002). Although it has been hypothesized that cichlids might represent an exception to this rule (Liem, 1980), recent evidence suggests that resource partitioning might be common even in hyperdiverse cichlid assemblages (e.g. Albertson, 2008; Arnegard, 2009; Danley, 2011; Hulsey *et al.*, 2013; Kerschbaumer *et al.*, 2014). Closely related, sympatric species of Lake Malawi cich-

lids have been shown to partition their habitat by depth (Albertson, 2008), along the benthic–limnetic axes (Hulsey *et al.*, 2013), and possibly by microhabitat (Danley, 2011), but not diet (Martin & Genner, 2009). Thus, habitat partitioning appears an important mechanism to avoid competition with congenerics in the rock-dwelling cichlids of Lake Malawi.

Building on a previous study that suggested microhabitat partitioning between a pair of barred and nonbarred *Maylandia* species (Danley, 2011), our study examined replicate species pairs to determine whether sympatric species diverged in body shape. At each sympatric location, both species were morphologically differentiated from each other. Interestingly, the direction of shape change was similar at all locations (Fig. 4, Table 3). The nonbarred species consistently had lower body depth and were more fusiform in comparison with the barred species. Such differences in body depth generally are associated with swimming performance in fish. A shallower, fusiform body allows for greater sustained swimming performance and therefore can be advantageous in open habitats, whereas a deeper body allows for higher manoeuverability and is advantageous in habitats with complex structures (e.g. Barlow, 1972; Webb, 1984; Langerhans & Reznick, 2009; Ruehl *et al.*, 2011).

This finding is consistent with previous behavioural studies. Danley (2011) found that *M. zebra* prefer cobble-rich habitat patches, whereas the nonbarred *M. benetos* prefer bedrock at Mazinzi Reef. Similar preferences for simple and complex habitats are found for *M. chrysomallos* and *M. zebra* at Chiofu Bay (P.D. Danley & M. Husemann, unpublished). Holzberg (1978) also documented differences in the preference of territory size and feeding habitat in the sympatric *M. zebra* and *M. callainos* at Nkhata Bay. In our study, it appears that

congeneric sympatric species have adapted to different microhabitats with deep-bodied species common in cobble-rich habitats and slender-bodied species common in structurally simpler habitats. The observed divergence in body shape thus may be adaptive to specific microhabitats and could therefore facilitate partitioning of the habitat to minimize competition for both food and territories (Webb, 1984).

Whereas male coloration has been clearly shown to be a sexual trait in Lake Malawi cichlids (e.g. Couldridge & Alexander, 2002; Kidd *et al.*, 2006), it additionally may provide an adaptive advantage in their respective microenvironments. Generally, in Lake Malawi's rock-dwelling cichlids, coloration is thought to be cryptic in females yet conspicuous in males (Roberts *et al.*, 2009). However, a meta-analysis by Seehausen *et al.* (1999) found that barred patterns are generally associated with complex habitats in cichlids (e.g. rocky or vegetated) and suggested an adaptive function of the barred pattern (but see Deutsch, 1997 for an alternate interpretation). If an interrupted colour pattern is an adaptation to complex habitats, the non-barred phenotype might be more cryptic over homogeneous habitats such as bedrock. Whereas an explicit test of the adaptive benefit of the barred and nonbarred phenotypes is still needed, we suggest that both coloration and body shape might be under correlational selection in response to mbuna microhabitat preferences (Brodie, 1992; McGlothlin *et al.*, 2005; Bergstrom, 2007; Carlsbeek & Irschick, 2007; Roff & Fairbain, 2012). Coloration, therefore, might serve dual purpose: male coloration likely plays an important role in reproductive interactions, while also providing camouflage in the preferred microhabitat.

We further found that the unbarred species are not monophyletic. This would indicate that the unbarred phenotype, as well as the associated body shape, has evolved multiple times independently possibly via parallel speciation. A recent study by Bierne *et al.* (2013), however, suggested that using nonselected genetic markers to investigate the potential for parallel speciation is nonconclusive due to potential introgression after secondary contact. Therefore, our result needs to be treated with caution, and future studies should include functional genetic markers under selection as well.

Evolutionary processes driving the divergence in sympatry

Whereas both ecological and sexual traits are differentiated in sympatry, it remains unclear whether this is a result of character displacement or ecological sorting. Character displacement has shown to be the driver of rapid phenotypic divergence in other systems of closely related, sympatric species (e.g. Adams, 2010; Scott & Johnson, 2010; Magalhaes *et al.*, 2012). The most well-

known examples are represented by the divergence of three-spined sticklebacks (Schluter & McPhail, 1992; Pritchard & Schluter, 2001) and Nicaraguan crater lake cichlids (Barluenga *et al.*, 2006) into limnetic and benthic forms, beak size divergence in Galapagos finches (Schluter & Grant, 1984; Grant & Grant, 2006), and ecomorph divergence in sympatric species of *Anolis* lizards (Losos *et al.*, 1998). If character displacement has driven this divergence, one would expect that nonsympatric populations of *M. zebra* occupy more morphospace than sympatric populations (Willis *et al.*, 2005), which is, in fact, the pattern observed in our data (Appendix S3). Furthermore, nonsympatric populations of *M. zebra* often have a more fusiform body shape compared with sympatric populations (Fig. 5). Whereas this pattern is consistent with ecological character displacement, explicit tests using manipulative approaches are needed to affirm that ecological character displacement caused the observed differentiation (Schluter, 2000; Stuart & Losos, 2013).

The alternative, ecological sorting, could also explain the consistent phenotypic differences observed between the sympatric species pairs (Armbruster *et al.*, 1994; Schluter, 2000; Sax *et al.*, 2007; Dangles *et al.*, 2008). Ecological sorting occurs when species are competitively excluded from a location if they are not pre-adapted to coexistence. This process can generate communities of ecologically divergent species capable of coexisting (Losos, 1990; Armbruster *et al.*, 1994; Schluter, 2000). Repeated environmental changes leading to range reductions and expansions provide the opportunity for ecological sorting to happen. Given the dynamic nature of Lake Malawi's water level, it is possible that ecological sorting has produced the observed phenotypic pattern. Throughout its history, Lake Malawi experienced multiple desiccation/inundation events producing cycles of admixture and separation of complex, geographically distinct cichlid communities (Danley *et al.*, 2012). However, ecological sorting suggests that species are static units that only survive in sympatry if they are pre-adapted to specific niches, which are not occupied by others (Armbruster *et al.*, 1994). Yet, our data suggest that *M. zebra* populations have experienced site-specific shape changes as response to local selective pressures and in response to the absence or presence of the congener rendering ecological character displacement the more likely explanation for the observed patterns.

Intraspecific population divergence

Whereas phenotypic differentiation is most apparent between *M. zebra* and the nonbarred species, this study also revealed morphological differentiation within *M. zebra*. Among East African cichlids, geographical separation and local adaptation often result in strong phenotypic and genetic differentiation (e.g. Bouton *et al.*, 1999, 2002; Streelman *et al.*, 2007; Postl *et al.*,

2008; Kerschbaumer *et al.*, 2011; Magalhaes *et al.*, 2012; Spreitzer *et al.*, 2012). Our results are consistent with these previous studies: *M. zebra* is morphologically differentiated for almost all of the analysed populations. The only exceptions are the geographically close populations of Otter Point and Illala Gap whose morphological similarity is likely the result of ongoing gene flow, which is supported by the extensive sharing of haplotypes (Fig. 6) and the low estimates of genetic divergence for these populations (Table 2). Among populations that are more distantly separated, however, no isolation by distance was found for either the genetic or the morphological data set. This suggests that geographical distance does not determine morphological or genetic similarity. Instead, the morphological differentiation of *M. zebra* populations is likely the result of local selective pressures. A similar pattern of local adaptation is seen in each of the nonbarred species (Fig. 4) and has been shown for other morphological characters in *M. zebra* (Streelman *et al.*, 2007), further underlining the important effects of the local environment on ecomorphology.

Conclusions

In this study, we show that consistent differences in an ecological trait exist in replicated sympatric species pairs of the rock-dwelling genus *Maylandia*. Morphospace occupation is smaller in sympatric populations than in nonsympatric populations suggesting that ecological character displacement might play a role for divergence. Coloration and body shape appear to have evolved in specific combinations likely as an adaptation to specific microhabitats potentially resulting from correlational selection. Allopatric populations of *M. zebra* are molecularly and phenotypically differentiated as result of geographical isolation and local selection. The divergence of *Maylandia* populations appears to be strongly driven by local adaptation as well as by sympatry with closely related species. The correlation of ecologically selected traits such as body shape with traits involved in reproduction may facilitate the coexistence of congers and help to generate and maintain species diversity in this system.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Densitree visualization of all trees resulting from *BEAST analysis.

Appendix S2 Re-analyses of AFLP data from Allender *et al.* (2003).

Appendix S3 Comparative analyses of morphospace across all sampled populations.