Genetic and morphological analyses indicate high population mixing in the endangered cichlid Alcolapia *flock of East Africa*

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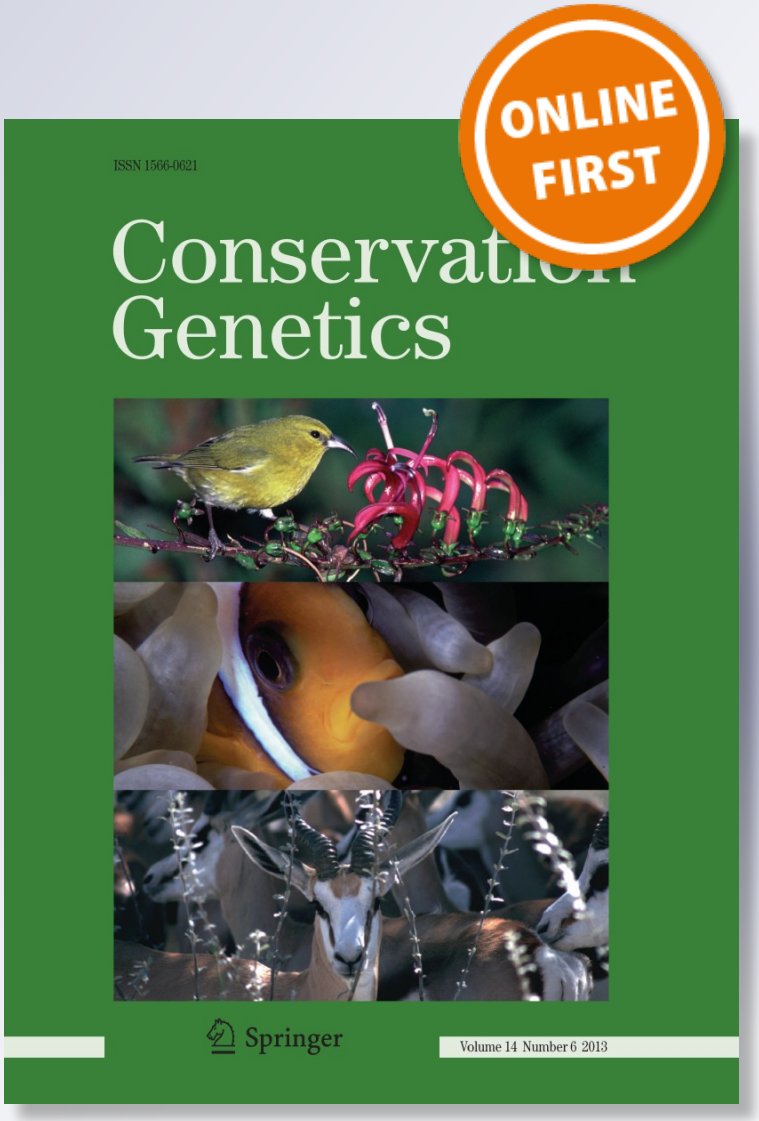

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RESEARCH ARTICLE

Genetic and morphological analyses indicate high population mixing in the endangered cichlid *Alcolapia* flock of East Africa

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Abstract *Alcolapia* is a minor genus of small-bodied, polymorphic cichlids inhabiting the lagoons and hot springs surrounding the soda lakes Natron (largely in Tanzania) and Magadi (Kenya). Three *Alcolapia* species are present at Natron (*Alcolapia alcalicus, Alcolapia ndalalani* and *Alcolapia latilabris*) and one at Magadi (*Alcolapia grahami*). All are IUCN Red Listed as either vulnerable or endangered. We performed analyses of morphometric and genetic structure on 13 populations of the Natron *Alcolapia* flock, and one *A. grahami* population of Lake Magadi as an out-group. Morphometric analyses revealed significant differentiation in the head and mouth shape of the species at Natron. From a genetic perspective, among 70 mtDNA control region sequences 17 haplotypes were found, showing in the minimum spanning network a star-like pattern around the widespread haplotype *2lat*. At Natron, there was limited genetic differentiation between the different populations of *A. alcalicus* and *A. latilabris*, despite apparent ecological barriers of extreme alkalinity that suggested their populations were isolated. Instead, there appeared to be some population connectivity, with a rate of 0.5–2.3 migrants per generation suggesting that natural factors, such as intense rains or transmission by large piscivorous birds, facilitate population connectivity and maintain genetic similarity. The outputs of high population connectivity and one genetic unit at the basin level (despite morphological divergence) suggest that any human activities that disrupt the connectivity of the freshwater resources of the Natron catchment could further threaten the integrity and current status of these already threatened fish populations.

Keywords *Alcolapia* · Gene flow · Extreme habitat · Cichlids · Morphometric analysis

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Introduction

Habitat change is a major cause of population decline for many threatened and endangered species (Duncan and Lockwood 2001; Fagan et al. 2002) through its consequences for population structure and effective size (Salgueiro et al. 2003; Laroche and Durand 2004; Salducci et al. 2005). Most genetic studies in freshwater fish focus on phylogeography and relate patterns to long-term

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historical events such as drainage system formation, including glaciations and historic climate variation (e.g. Culling et al. 2006; Gagnon and Angers 2006). From a conservation perspective, these studies are important as they provide recommendations for appropriate management and conservation plans that seek to protect threatened species (Sousa et al. 2010). Genetic tools can also resolve taxonomic problems for species that are difficult to identify, detect differentiation within and between geographical populations, and identify patterns of gene flow (Vrijenhoek 1998).

Alcolapia is a genus of small, polymorphic cichlid fishes that is restricted to the freshwaters of saline-alkaline lakes in East Africa. Four morphotypes have been identified and are naturally confined to Lakes Magadi (Kenya) and Natron (Tanzania) (Coe 1966, 1969). These two saline-alkaline (colloquially 'soda') lakes are shallow, warm (40–44 °C), rich in dissolved sodium salts (especially sodium carbonate/bicarbonate, chloride, and sulphate) and are characterized by caustic conditions (pH $>$ 10; conductivity $\geq$107,000 μS cm^{-1}). These conditions produce deposits of sodium carbonates called trona ($Na_3H(CO_3)_2$) at commercially-exploitable quantities (Reite et al. 1974; Seegers and Tichy 1999). There is a wide spectrum of ecological adaptations and tolerances to pH, temperature and salinity within *Alcolapia* species that arise from their unique biological and physiological features (Wilson et al. 2000; 2004), their presence is restricted to the less saline springs and streams surrounding the lakes (Tichy and Seegers 1999). They are rarely observed in the open water of the lakes, where shallow waters create harsh conditions (high alkalinity and temperatures up to 44 °C; Seegers and Tichy 1999). A recent taxonomic revision of the *Alcolapia* described four morphotypes, considering them as distinct species according to their morphological and ecological parameters (Seegers and Tichy 1999). *Alcolapia grahami* are encountered only in the Lake Magadi springs (Seegers and Tichy 1999) whereas *Alcolapia alcalicus, Alcolapia ndalalani* and *Alcolapia latilabris* are confined to the edges of Lake Natron (Seegers and Tichy 1999) where they are present in hot springs and streams. In these habitats, there is a general tendency for their habitat partitioning; *A. latilabris* is present primarily in the upper, warmer reaches of the springs whereas *A. alcalicus* is more abundant in the lower reaches and lagoons. However, their physiological adaptations to extreme environments might enable them to share similar habitats during, for example, periods of flooding (Tichy and Seegers 1999). This would then potentially influence population genetic structure and the genetic differentiation within the flock that has been described as a unique phylogenetic unit (Seegers et al. 1999).

All four *Alcolapia* species are on the IUCN Red List; at Lake Natron, *A. alcalicus* is 'endangered' (Bayona 2006) while both *A. ndalalani* and *A. latilabris* are 'vulnerable'. *A. grahami* (Boulenger 1912) is 'vulnerable' at Magadi (IUCN 2012). A particular threat is habitat disruption caused by soda ash mining that continues in Magadi and is proposed as an industrial development in Natron. The latter is also affected by catchment-wide use of freshwater run-off for irrigation, cattle watering and domestic needs. Future plans also include the development of hydro-power in the Ewaso Ngiro catchment of Natron. These developments raise conservation concerns in relation not only to the fish but also to the only flamingo breeding site in East Africa (Brown and Root 1971), through hydro-chemical disruptions that might occur.

Information on species populations and population structure is necessary to effectively design conservation programmes for the protection of the *Alcolapia* genus. As such, the objectives of this study were to (i) quantify the genetic and morphological characteristics of the four *Alcolapia* species; (ii) evaluate their population structures and assess patterns of gene flow between the Lake Natron populations; and (iii) use the outputs from (i) and (ii) to assess the current status of the *Alcolapia* in the two lakes to assist indication of conservation priorities and develop appropriate strategies. These objectives were completed using mitochondrial and microsatellite markers to investigate the genetic differentiation across the two catchments, complemented by morphometric analyses at Lake Natron that assessed the morphological differentiation between the species.

Materials and methods

Study catchments

Lake Natron and Lake Magadi are in East Africa's Rift Valley (Fig. 1). Lake Magadi (1°50′ S, 36°18′ E, 600 m.a.s.l.) is ~19 × 6 km in size (Maina et al. 1996) and is a closed alkaline lake at Kenya's southern end of the Rift Valley. It is hypersaline-alkaline, <1 m deep with a trona crust, and fed almost entirely by groundwater inflows through springs whose pH is 9.0–9.4 (Roberts et al. 1993). Lake Natron, whose northern extent is situated 20 km south of Magadi, is located between 2°09′ and 2°35′ S and 35°53′ and 36°06′ E at 608 m a.s.l. It is ~60 × 17 km in size, covering around 1,039 km^2 (Page and Simon 1987). It is shallow and hydrologically variable (Tebbs 2013) with its central part occasionally consisting of wide soda mud flats. ~50 % of its inflow is received from rivers, 25 % from springs and 25 % from direct rainfall. The major rivers include the Engaresero, Monik, Pinyinyi (in Tanzania) and the Ewaso Ngiro South (in Kenya). These rivers and some of the springs support near-permanent shallow

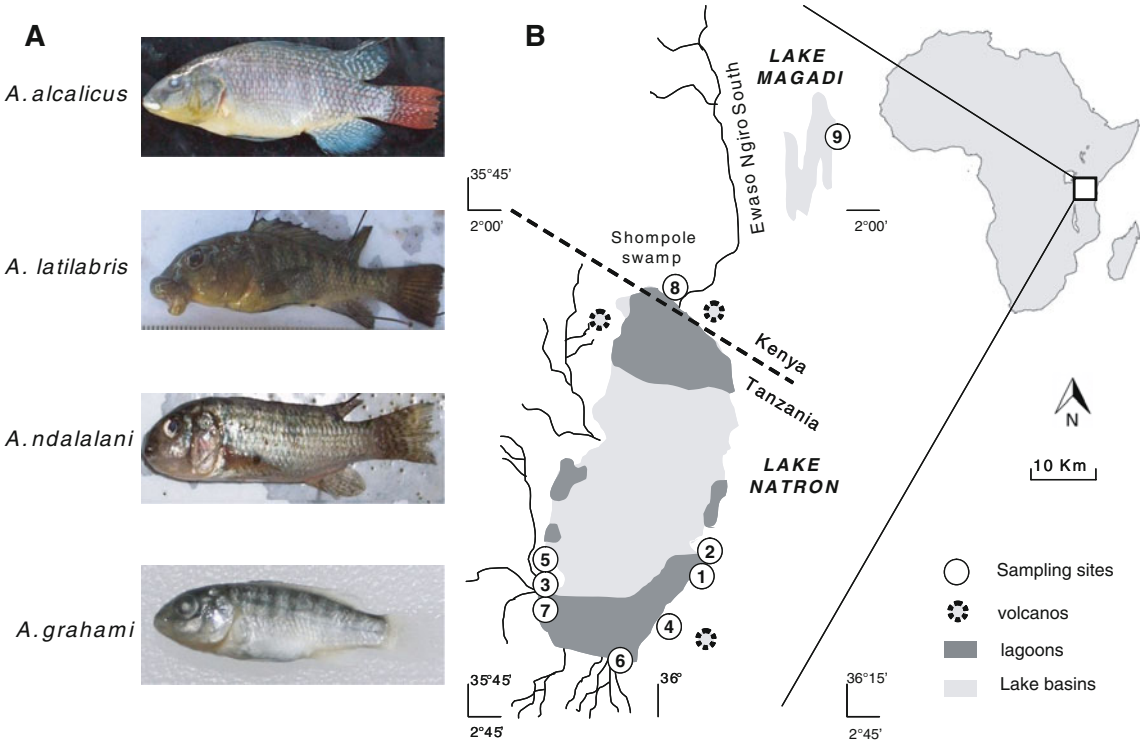

Fig. 1 The *Alcolapia* flock species and the nine sampling localities (cf. Table 1). The geographic features of basins (e.g. main lake, near-permanent shallow lagoons around the edge of the lake, and volcanoes) are also indicated (modified from Seegers et al. 1999). The lagoon areas (*dark grey*) are subjected to size variation according to rains

lagoons around the edge of the lake of lower alkalinity, which sometimes hold *Alcolapia* populations that have spread from the springs supporting water birds including pelicans as well as lesser flamingos.

Fish sampling

Fourteen populations of the *Alcolapia* flock were sampled in April 2009 from eight sampling sites at Lake Natron and one at Magadi (Fig. 1; Table 1). Thirteen populations were sampled at Lake Natron: four populations of *A. latilabris*, two populations of *A. ndalalani* and six populations of *A. alcalicus*, while a single *A. grahami* population from Magadi was used as an outgroup. At Natron, *A. latilabris, A. ndalalani* and *A. alcalicus* were sympatric at two sampling sites (Loc4 and Loc6) in two lower reaches close to the south permanent lagoon of lake Natron, while *A. latilabris* and *A. alcalicus* were also sampled together in one reach (Loc7) in the south-western side (Table 1; Fig. 1). Fish samples were collected using a combination of scoop, seine and cast netting (method dependent upon habitat). Individuals were identified in the field according to their morphological description, particularly their mouth morphology, according to the descriptions of Tichy and Seegers (1999) and Seegers and Tichy (1999). Prior to their release, a random sub-sample of each species was taken (maximum sample size (n) of 30 per species). Individuals were anaesthetised using clove oil, photographed for subsequent morphological analyses and a tissue sample ($n = 30$ pelvic fin/species/site) collected and stored in 98 % ethanol.

Fish morphometrics

The morphological traits were measured on each sampled fish ($n = 30$ species/site) to the nearest 0.001 mm from digital images using IMAGE J (U.S. National Institute of Health, http://rsb.info.nih.gov/ij/). The measured traits were a combination of 25 measurements specific to the head (Seegers and Tichy 1999; Maldonado et al. 2009) and 11 general body morphology traits (Blanchet et al. 2008). Regression analysis was then used to identify the presence of collinear traits; where tolerance (T) was <0.1 then the traits were considered collinear and were eliminated from subsequent analyses (Belsey 1991). This procedure yielded 12 morphological traits that were related to differences in body shape between species; five related to head morphology (head height, maxillary length, pre-orbital distance, orbital diameter and head length) and seven to body morphology (pre-pelvic length, length of dorsal fin base, distance of the origins of dorsal and anal fins, length of anal

Table 1 Sampled populations are detailed for *Alcolapia* species; sampling sites with geographic coordinates (from Loc1 to Loc8 at lake Natron; Loc9 at lake Magadi, as presented in Fig. 1)

Sampling sites	S	E	Pop code	*Alcolapia* species	NA	A_R	H_e	H_o
Loc1	02 27.366	036 05.287	Pop1	*A. latilabris*	17	7.6	0.505	0.434
Loc2	02 26.081	036 05.873	Pop2	*A. alcalicus*	16	8.1	0.626	0.512
Loc3	02 30.653	035 53.144	Pop3	*A. alcalicus*	32	9.1	0.615	0.529
			Pop4	*A. alcalicus*	16	7.7	0.529	0.374
Loc4	02 31.629	036 02.762	Pop5	*A. latilabris*	16	7.7	0.517	0.441
			Pop6	*A. ndalalani*	9	5.4	0.519	0.474
Loc5	02 27.353	035 53.478	Pop7	*A. alcalicus*	34	11.1	0.616	0.501
			Pop8	*A. alcalicus*	31	11.0	0.577	0.543
Loc6	02 35.415	036 00.427	Pop9	*A. latilabris*	31	9.9	0.499	0.407
			Pop10	*A. ndalalani*	27	10.1	0.575	0.552
Loc7	02 35.400	035 55.117	Pop11	*A. alcalicus*	31	10.0	0.563	0.455
			Pop12	*A. latilabris*	14	5.6	0.490	0.404
Loc8	02 06.495	036 00.379	Pop13	*A. alcalicus*	21	9.1	0.622	0.654
Loc9	01 53.270	036 18.494	Pop14	*A. grahami*	15	7.1	0.582	0.58

Genetic diversity indices averaged over seven microsatellite loci are reported for 14 populations, detailing NA (sample size), mean expected and observed heterozygosity (H_e and H_o, respectively) and A_R (allelic richness)

fin base, pre-dorsal length, caudal peduncle length and caudal peduncle depth). The relationship between total length and each morphometric trait was tested for significance using Pearson's Correlation Coefficient; where significance indicated an allometric effect of body size on the morphometric trait ($P < 0.05$), it was used in Eq. 1 (Lleonart et al. 2000):

$$M_i^* = M_i(TL_0/TL_i)^b \quad (1)$$

M_i^* is the allometrically-adjusted morphometric trait, M_i is the original morphometric trait, TL_0 is the mean total length of all fish in sample, TL_i is the total length of individual fish, b is the regression coefficient of the relationship between total length and morphometric trait of fish in the sample.

The relationship between fish length and the trait was re-tested after transformation; where the relationship was not significant, then the allometric effect was considered as removed and appropriate to use in subsequent analyses. Differences in body shape between the species were then analysed using ANOVA, principal components analysis (PCA) and discriminant function analysis (DFA). ANOVA and post hoc tests revealed the traits that were significantly different between species; PCA and DFA were used to determine the number of species that could be identified within the data according to their morphology. DFA was completed using cross–validation using the leave-one-out method of classification. These analyses were used initially at a single site where the species were present in sympatry and then using data from all of the species from across the basin (Table 1).

CR mtDNA sequencing and diversity

A 350 bp sequence of control region (CR) was amplified for 70 *Alcolapia* individuals (Table 2). Forty-one sequences were completed for *A. alcalicus* from seven sample sites at Lake Natron (with the exception of Loc1), five sequences for *A. ndalalani* collected from two sample sites (Loc4 and Loc6), and eighteen sequences for *A. latilabris* from four sample sites. Six sequences for *A. grahami* from Lake Magadi were also included (Loc9). The CR marker was selected as studies have indicated high levels of polymorphism in tilapiines (e.g. Seegers et al. 1999; Nagl et al. 2001). Primers selected for the amplification were L-15926 (Seegers et al. 1999) and Ormt-917LP (Nagl et al. 2001). Amplification was carried out in 25 µl reaction volume that contained one unit of Taq DNA Polymerase (Sigma-Aldrich, Germany), 1X reaction buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP and 12.5 pmol of each primer. The reaction was run under the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 60 s and extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. Template DNA was purified using the Montage PCR96 Cleanup Kit (Millipore). Purified PCR product (2 µl) was used as the template in the cycle sequencing reaction with L-15926 primer and Dynamic ET Dye Terminator Cycle Sequencing Kits (Amersham, Biosciences). The cycle-sequenced products were analysed on an automated capillary DNA sequencer (500 Megabase, GE Healthcare). The DNA sequences were then edited and aligned manually using Bioedit software

Table 2 Haplotype distribution of CR mtDNA fragment (350 bp long) for the 14 populations are listed

Pop	Species	Loc	N	Haplotype distribution															
				2lat	3lat	53	50	68	70	305	7	42	241	243	398	238	125	34	1	3
Pop1	*Al*	Loc1	4	4																
Pop3	*Aa*	Loc3	13	4	7			1	1											
Pop4	*Aa*		4	3						1										
Pop5	*Al*	Loc4	2	2																
Pop6	*An*		1	1																
Pop7	*Aa*	Loc5	5	4													1			
Pop8	*Aa*		7	4									1	1		1				
Pop9	*Al*	Loc6	7	6											1					
Pop10	*An*		4	3	1															
Pop11	*Aa*	Loc7	7	3		1	2					1								
Pop12	*Al*		5	2	2						1									
Pop13	*Aa*	Loc8	5	3	2															
Pop14	*Ag*	Loc9	6	3														1	1	1
Total			70	42	12	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1

Population code, *Alcolapia* species (*Aa* = *A. alcalicus*; *Al* = *A. latilabris*; *An* = *A. ndalalani*; *Ag* = *A. grahami*), sampling sites (Loc), sampling size (N) are detailed. For sampling site 2 (loc2) (*A. alcalicus)* no sequences were included

(Hall 2003). A minimum-spanning network, coupled with statistical parsimony analysis using the TCS program (Clement et al. 2000), was then constructed.

Microsatellite typing and diversity

Three hundred and ten fish (181 *A. alcalicus*, 36 *A. ndalalani*, 78 *A. latilabris* and 15 *A. grahami*) were amplified for seven microsatellite loci designed for *Oreochromis niloticus* (UNH843, UNH851, UNH874, UNH891, UNH958, UNH989 and UNH915) (Carleton et al. 2002). Amplifications were performed using a Mastercycler EPgradient thermal cycler (Eppendorf). Fluorescent labelling of PCR fragments was carried out using 6-FAM, PET, VIC and NED dyes (Schuelke 2000). PCR amplifications were performed in a final volume of 20 µL containing 10 ng of genomic DNA, 0.5 units of GoTAQ Flexi DNA Polymerase (Promega), 1X reaction buffer, 1.5 or 2 mM of $MgCl_2$ (1.5 mM for UNH989; 2 mM for all other primers), 200 µM of each dNTP (Promega), 3.2 pmol of each reverse and labelled-M13(-21) primer and 0.8 pmol of the forward primer. The reaction was run under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at Ta °C for 45 s and extension at 72 °C for 45 s, followed by 8 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s and at 72 °C for 45 s, and final extension at 72 °C for 10 min. The Ta used was: Ta = 55 °C for UNH843, UNH851, UNH891, UNH915, UNH958; Ta = 57 °C for UNH989; and Ta = 59 °C for UNH874. The amplification products were run on a capillary ABI3730XL sequencer (Applied Biosystems) and the genotypes were obtained after scoring the raw data with the Peak Scanner™ Software Version 1.0 (Applied Biosystems) to score the single-fish genotypes.

Levels of genetic variability, including allelic richness (A_R), observed (H_o) and expected (H_e) heterozygosities, were estimated for all 14 populations. Deviation from the Hardy–Weinberg equilibrium was also tested by a Fisher's exact test using the Markov Chain algorithm (Guo and Thompson 1992). The software used was GENEPOP (Raymond and Rousset 1995) and Genetix (Belkhir et al. 1996).

Population genetic structure analyses

The extent of genetic differentiation was tested among the populations. Values of F_{ST} (Weir and Cockerham 1984) were computed using Genetix; the significance of F_{ST} values was tested by a permutation procedure and Bonferroni correction (Rice 1989). Isolation by distance (IBD) was investigated using the Mantel test (Mantel 1967), implemented in GENEPOP, by comparing pairwise genetic distances calculated from $F_{ST}/(1-F_{ST})$ with the natural logarithm of geographic distances between the Natron sample localities. Two possible dispersal routes were tested: i) the 'shorter' route across open water during intense flood conditions, in which the high water and diluted alkalinity would enable the fish migration between the isolated localities (i.e. south and north lagoons) or over the lake by birds; and ii) the longer route 'via the shoreline', along the periphery lagoons around the main lake.

Gene flow ($4N_em$) and theta values ($\Theta = 4N_e\mu$) were then estimated among populations of *A. alcalicus* and *A. latilabris* using the coalescent approach implemented in MIGRATE (Beerli and Felsenstein 2001; Beerli 2007). For each run, 10 short chains of 500 sampled and 100 recorded trees, followed by one long chain of 5,000 sampled and 100 recorded trees were used, with a 1,000 tree burn-in. The full migration model was used and assumed symmetrical gene flow. The estimates of Θ and gene flow were generated from F_{ST} values adopting the maximum-likelihood inference (95 % confidence intervals). We used the Bayesian clustering method implemented in the software STRUCTURE 2.1 (Pritchard et al. 2000) to identify the most probable number of genetic units (K). This was applied to the entire dataset (14 populations) and also to two subsets of data (*A. alcalicus* and *A. latilabris* species). Twenty runs for each K value from 1 to 10 were performed to assess consistency of likelihood estimations. Each run consisted of 10^5 MCMC iterations with an initial burn-in of 2×10^4 steps. The admixture model with correlated allele frequencies (Falush et al. 2003) was chosen since there were no a priori reasons to exclude mixed ancestry of individuals and the results strongly suggested the current species were derived from the same ancestral population. The most likely K was evaluated by ΔK, the second-order rate of change of the likelihood function with respect to K (Evanno et al. 2005), using STRUCTURE HARVESTER to visualize the outputs (Dent and von Holdt 2012).

Demographic analysis

Historical demographic analyses for CR mtDNA data were performed on *A. alcalicus* and *A. latilabris* at Natron. Fu's Fs (Fu 1997) and Tajima's D (Tajima 1989a, b) tests were included. Molecular diversity was estimated by the haplotype diversity (H) and the nucleotide diversity (π). Mismatch distribution and raggedness index were performed (Rogers and Harpending 1992, Harpending et al. 1993), testing the sudden expansion model (Rogers 1995). All analyses were computed with ARLEQUIN 3.1 (Excoffier et al. 2005) and DnaSP 5.10 (Librado and Rozas 2009).

Results

Fish morphometric analysis

The allometric transformation (Eq. 1) removed the significant effects of body size from the morphological traits. At sampling site 4 (Loc4), where the Natron *Alcolapia* spp. were present in sympatry, ANOVA revealed significant differences between the three species in all of the transformed morphological traits ($P < 0.05$) except orbital diameter ($F_{2,88} = 1.77$; $P > 0.05$). ANOVA Tukey post hoc tests revealed the highest morphological divergence between *A. alcalicus* and *A. latilabris*, with significant differences for 10 traits ($P < 0.05$; Supplementary Material Annex Table A1). A PCA of the significant morphological data (i.e. omitting orbital diameter) revealed that PCI explained 78 % and PCII 13 % of the morphological variation between the species (Fig. 2a). *A. alcalicus* and *A. latilabris* displayed the highest differentiation when the individual scores were plotted in the space of the first two principal components. The significantly different morphological traits were then used to train a discriminant function that successfully classified three groups in which the first canonical discriminant function explained 100 % of variance and separated the fish by species (Wilks

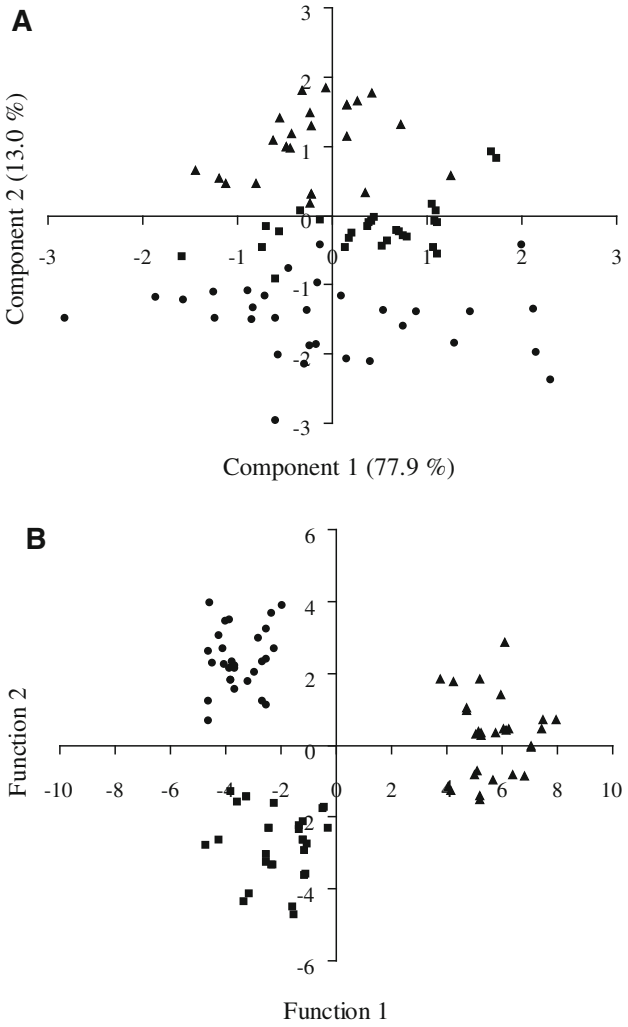

Fig. 2 Morphometric analyses at sampling site 4 (Loc4). **a** Scatterplots of individual fish in the morphospace along principal components I and II; **b** Plot of Function 1 against Function 2 of discriminant analysis for the *Alcolapia* fishes in Loc4. *filled triangle* = *A. latilabris*; *filled square* = *A. ndalalani*; *filled circle* = *A. alcalicus*

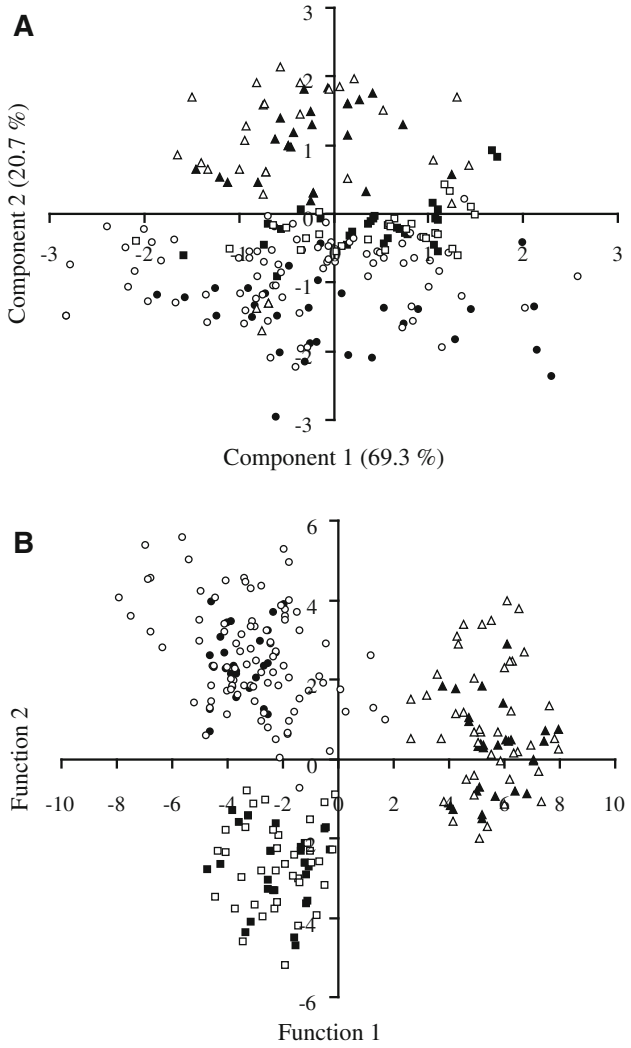

Fig. 3 Morphometric analyses at Natron basin. **a** Scatter-plots of individual fish in the morphospace along principal components I and II; **b** Plot of Function 1 against Function 2 of discriminant analysis for the *Alcolapia* fishes in Loc4 and the predicted group membership for the species from the rest of the basin. *filled triangle* = *A. latilabris,* Loc4; *open triangle* = *A. latilabris*, rest of basin; *filled square* = *A. ndalalani*, Loc4; *open square* = *A. ndalalani*, rest of basin; *filled circle* = *A. alcalicus*, Loc4; *open circle* = *A. alcalicus,* rest of basin

Lambda = 0.232; $P < 0.01$). The cross-validation procedure correctly classified all of the fish according to their identified species (Fig. 2b). Thus, the morphology of the fishes enabled their differentiation at an individual site.

Morphology data from all of the sampling sites were significantly different in the traits of *A. alcalicus* and *A. latilabris* ($P < 0.05$) except orbital diameter ($F_{2,202} = 0.97$; $P > 0.05$). PCA revealed that PCI explained 69 % and PCII 21 % of the morphological variation between the species (Fig. 3). The trained discriminant function model from Loc4 then successfully predicted the presence of the three species, with 98 % of the specimens successfully classified into their identified species. Thus, the morphology of the fishes enabled their differentiation by species at the basin level (Fig. 3).

mtDNA variation

Eighteen polymorphic sites were found for the 350 bp length of the mtDNA CR and defined 17 haplotypes (Table 2, GenBank Acc. Nos. HQ637440-HQ637456) that differed by 1–11 nucleotides. These haplotypes showed a star-like pattern when analysed by the Minimum Spanning Network (MSN; Fig. 4). The haplotype *2lat,* displaying a central position in the MSN, was abundant, being found in 42 individuals and being widespread in all sampled localities and in every species. Haplotype *3lat*, different from *2lat* at one nucleotide, was widespread only in the Natron flock and especially in *A. alcalicus* (75 % of the tested fish).

Nuclear DNA genetic variability

There were 147 distinct alleles in 7 microsatellites within the 310 analysed fish. The total number of alleles per locus varied from 3 (locus UNH851) to 48 (locus UNH989). Microsatellite diversity indices per population (Table 2) revealed the genetic diversity (Nei's heterozygosity, He) was medium for each population, ranging from 0.490 (Pop12) to 0.626 (Pop2). Only three populations (Pop1, Pop6 and Pop14) were found to be at Hardy–Weinberg equilibrium, while an excess of homozygotes was present in 11 populations, showing departures from Hardy–Weinberg equilibrium.

Genetic population structure

Whilst genetic differentiation was low across all of the species ($F_{ST} = 0.044$), between the 14 populations there was significant genetic differentiation in 57 of 91 pairwise comparisons ($P < 0.05$; cf. Supplementary Material Appendix Table A2). The pairwise F_{ST} values among the Lake Natron populations (Pop1 to Pop13) and the Magadi population (Pop14) were significant ($P < 0.05$ after Bonferroni correction for multiple comparisons) with the only exception between Pop14 (*A. grahami*) and Pop2 (*A. alcalicus*), sampled in Loc2 at Lake Natron (Fig. 1). The significant pairwise F_{ST} values among Lake Natron species ranged from 0.03 (Pop10 *A. alcalicus*–Pop13 *A. ndalalani*) to 0.10 (Pop3 *A. alcalicus*–Pop9 *A. latilabris*). The significant pairwise F_{ST} values between sampling locations were between 0.02 (Loc3–Loc8 assessed within *A. alcalicus* populations) and 0.11 (Loc6–Loc7 assessed within *A. latilabris* populations). Significant genetic differentiation among the species in sympatry was found between *A.*

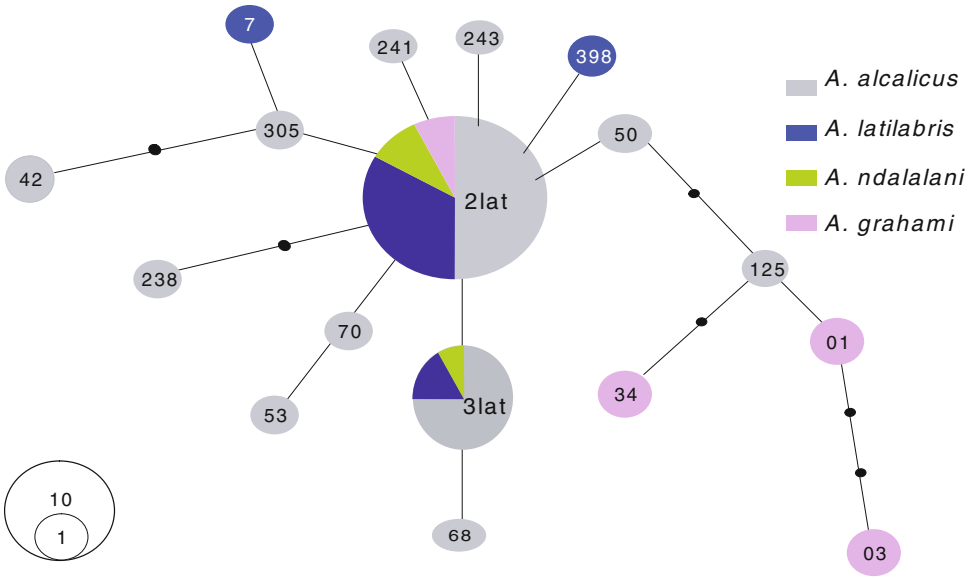

Fig. 4 Minimum spanning network of *Alcolapia* flock based on 350 bp long CR mtDNA sequences. Each *circle* represents one haplotype and the sizes of circles is proportional to the number of individuals sharing the same haplotype (see scale). *Small black circles* represent mutation steps. *Slices within circles* illustrate the haplotype distribution between the four species (distinguished with different colours)

alcalicus and *A. latilabris* (Pop8-Pop9), and between *A. latilabris* and *A. ndalalani* (Pop10-Pop9) at Loc6, a site characterised by a braided delta (Engare Sero River) (Fig. 1).

No significant correlations between genetic and geographic distances were found among *A. alcalicus* ($z = 3{,}458$, $g = 0.81$, $P = 0.314$; and $z = 2{,}722$, $g = 0.17$, $P = 0.52$ via shoreline and across open water respectively) or among *A. latilabris* populations ($z = 1.02$, $g = 1.038$, $P = 0.138$; and $z = 1.02$, $g = 1.036$, $P = 0.127$ via shoreline and across open water respectively). IBD was not applied to *A. ndalalani* due to the low number of populations sampled.

There was no clear signal in the clustering, even though the first Bayesian analysis, where the entire dataset was divided into 14 populations, indicated $K = 7$ as the most probable value. This suggested that the seven groups were unable to be attributed to any of the morphologically defined species with no evidence of genetic structuring (cf. Supplementary Material Annex Figure A1). A separate Bayesian clustering analysis that used only the fishes morphologically classified as *A. alcalicus* (181 individuals) and *A. latilabris* (78 individuals), also revealed a lack of structuring between the species.

Asymmetric migration for *A. alcalicus* and *A. latilabris* populations between the sampling locations was detected through the coalescent approach performed by MIGRATE (Supplementary Material Annex TableA3 and A4). The Θ values ranged from 1.62 to 3.93 and from 3.41 to 5.12 for each species respectively. Most confidence intervals of the estimated number of migrants overlapped and no differences in dispersion without asymmetric migration routes were found. The estimated number of migrants was similar for the two species, ranging from 0.48 (M_63) to 2.31 (M_52) for the *A. alcalicus* populations and from 0.62 (M_71) to 1.89 (M_41) for *A. latilabris*. The estimated number of migrants among the seven *A. alcalicus* populations and among the four *A. latilabris* populations, located around the southern lagoon (from Loc1 to Loc8), was approximately 1 migrant per generation, suggesting no barrier to dispersal (cf. Supplementary Material Appendix Table A3 and Table A4). The estimated number of migrants between the most distant *A. alcalicus* populations (Pop13–Loc8) ranged from 1.0 to 1.6 migrants per generation. The exception was the limited gene flow detected between Loc8 and Loc3 (M_83 = 0.73).

Demography of the Natron flock

The mismatch distribution of pairwise nucleotide differences for the two most abundant Lake Natron species, *A. alcalicus* and *A. latilabris*, produced distinctive unimodal curves that are characteristic of populations that have undergone a bottleneck followed by a sudden expansion (Fig. 5). This distribution did not deviate significantly from the expected distribution obtained under a sudden expansion model (*A. alcalicus*: SSD = 0.014, $P = 0.53$; Harpending's raggedness index $r = 0.07$, $P = 0.68$ and *A. latilabris*: SSD = 0.0002, $P = 0.90$; Harpending's raggedness index $r = 0.14$, $P = 0.82$).

Genetic diversity indices for *A. alcalicus* ($n = 41$) and *A. latilabris* ($n = 18$) ranged between 0.40 and 0.69 (haplotype diversity) and between 0.002 and 0.003 (nucleotide diversity). Fu's Fs and Tajima's D tests exhibited significant negative values for the Natron flock and for *A. alcalicus* species (Supplementary Material Appendix, Table A5).

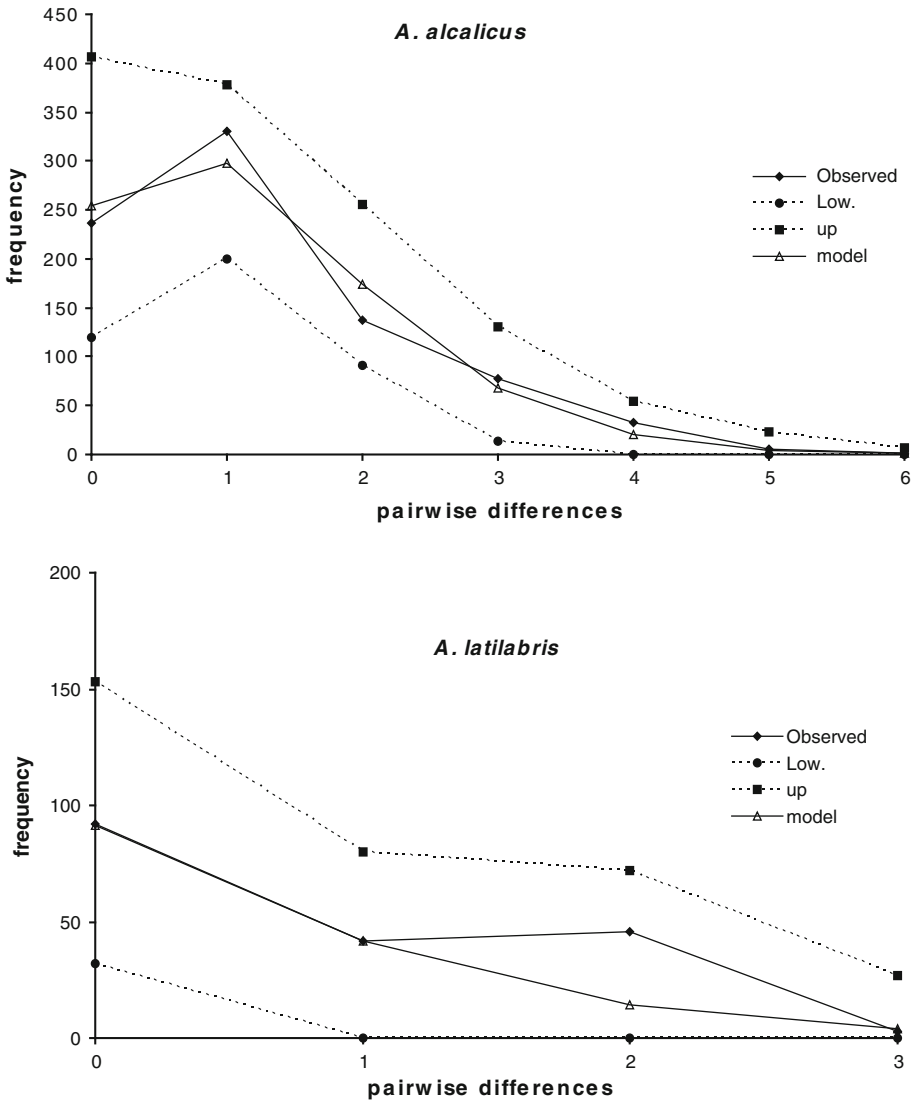

Fig. 5 Mismatch distribution based on CR mtDNA sequences for *A. alcalicus* and *A. latilabris*. Observed and expected distributions under the sudden expansion model of Rogers (1995) are reported. The *upper* and *lower bound curves* are 5 and 95 percentile values of 5,000 simulations

Discussion

Morphological diversity with genetic similarity

In the *Alcolapia* flock, the mitochondrial and nuclear data confirmed high levels of genetic homogeneity among the four species. This is consistent with other studies of East African cichlids that have revealed similar levels of genetic homogeneity and allele sharing between species or lineages with divergent phenotypes (Sturmbauer et al. 2001; Loh et al. 2008; Seehausen et al. 2008; Sylvester et al. 2010; Loh et al. 2011; Roberts et al. 2011). Conversely, the *Alcolapia* species were morphologically differentiated, particularly in mouth morphology, as also indicated by Tichy and Seegers (1999) and Seegers and Tichy (1999). This lack of concordance between the morphologic species and the underlying genetic unit might relate to differences arising from their foraging behaviours that has enabled resource partitioning and morphological divergence when in sympatry but that has been sufficiently recent such that it has not yet resulted in genetic differentiation.

In other cichlid flocks, the origin of morphological diversity tends to be recent adaptive radiations from a common ancestor (Joyce et al. 2005). This was also likely to be the case here, as during wetter periods in the Pleistocene, a larger lake occupied the whole basin in which Natron and Magadi both now sit (Baker 1958). The final appearance of this proto-lake, Lake Orolonga, was in the Holocene (9,000–10,000 years ago). Drying events and subsidence processes subsequently reduced its water level and formed the two separate lakes of Magadi and Natron, and perhaps also Manyara (35 km south from Lake Natron) (Hillaire-Marcel and Casanova 1986; Roberts et al. 1993). In Lake Orolonga, it appears that an *Alcolapia* flock emerged rapidly during a short, geologically transient window of opportunity (Tichy and Seegers 1999; Seegers

and Tichy 1999) and persisted after the geological and hydrological split between Natron and Magadi. This is reflected by the mitochondrial 'star phylogeny' of the ancestral haplotypes (the Orlonga haplotype) that was fixed at an early stage of the colonisation of Magadi and Natron (Seegers et al. 1999; Nagl et al. 2001) and is shared by all the *Alcolapia* species. When present in Orolonga, the *Alcolapia* might have been able to exploit a greater range of ecological niches that could have been provided by a larger lake and that subsequently provided them with high morphological and functional diversity. This scenario can be compared with the extinct Haplochromine species flocks that originated at Lake palaeo-Makgadikgadi in Southern Africa, that dried up in the Holocene. The centre of this extinct lake is now a saltpan north of the Kalahari Desert, but it once hosted a radiation of rapidly evolving fish species (Joyce et al. 2005). It reveals how local evolutionary processes operating during short windows of ecological opportunity can subsequently have substantial lasting effects on biodiversity at very large spatial scales.

Gene flow in Lake Natron

Levels of population genetic differentiation between the *Alcolapia* species are similar to F_{ST} values reported in several studies (based on SNPs) of closely related cichlid populations at Lake Malawi (Loh et al. 2008; Roberts et al. 2009) and Lake Victoria (Seehausen et al. 2008). According to the standing variation definition (Barrett and Schluter 2008), the sharing of genetic polymorphism may be facilitated not only by gene flow between populations but by several other factors including retention of ancestral polymorphism and/or hybridization phenomena (Loh et al. 2013). However, no hybrids have been sampled or documented in *Alcolapia* to date (cf. Seegers and Tichy 1999; Tichy and Seegers 1999, this study). The limited genetic differentiation detected among *Alcolapia* populations (detailed here for *A. alcalicus* and *A. latilabris*) was consistent with an effective rate of 0.5–2.3 migrants per generation between the populations and was supported by the lack of population genetic structuring. This may have favoured the sharing of allelic diversity in time and space as also depicted in the Lake Malawi and Tanganyika cichlid groups (Duftner et al. 2006; Loh et al. 2013).

This rate of migrants per generation might be surprising considering the main body of the lake is characterised by extreme saline and thermal conditions (conductivity to 107,000 μS cm^{-1}; temperatures to 44 °C) and presence of soda slush or islands. Such conditions would be expected to provide an effective barrier to fish migration but appear to be sufficiently permeable to allow some mixing. Thus, the apparent hostile habitat of the main lake only limits—not prevents—fish dispersal. As there is no evidence suggesting there are distinct dispersal routes, we suggest it might occur as a result of the occasional breaching of this 'barrier' during short periods of high rainfall that fills the lake basin, such as in March 2007 when the lake area was 804 km^2 (Landsat image 19/03/2007), compared to only 81 km^2 in October 2010 (Landsat image 25/10/200; Tebbs 2013). Moreover, piscivorous birds such as pelicans can potentially transport live fish, juveniles or eggs from one feeding ground to the next, and the inter-lagoon or cross-lake journey is no more than a few minutes flight, which could be sufficient time for a fish trapped in a beak pouch, feather or foot webbing to be released alive.

Conservation priorities in the Lake Natron basin

In general, conservation efforts should focus upon the preservation of overall biodiversity (based on morphological and genetic distinctiveness) and, concomitantly, protect landscapes and ecosystems that provide the essential life-support systems for endangered as well as non-endangered species. Knowledge about the population genetic structure and gene flow among populations is the first step in the conservation management of the *Alcolapia* flock, as also for several other threatened cichlid populations, particularly Haplochromines from lakes Victoria and Malawi (e.g. Kaufman and Ochumba 1993; Fiumera et al. 2000; Turner et al. 2001; Abila et al. 2008). The *Alcolapia* fishes, endemic to these alkaline-saline lakes were morphologically assigned to different "species", but the mtDNA and microsatellite analyses of samples collected from multiple locations within the basin did not show population structuring. This lack of concordance between the morphologically distinct species and their genetic unit suggests they are rapidly undergoing a species radiation. Moreover, the finding that some fishes do disperse across apparently hostile habitats highlights the importance of population connectivity at the basin scale. In combination, these outputs are important in developing the future conservation strategy of the lake basin, especially since management's main aim is to maintain its biodiversity richness. Such a strategy would need to address any issues that potentially threaten the ability of the fishes to disperse across the main lake and needs to preserve their genetic variability through managing the flock as one genetic unit. This suggests that any anthropogenic disturbances that ultimately reduce population connectivity and further fragment the freshwater habitats of the catchment should be strongly avoided. Consequently, any development projects must address the conservation of this *Alcolapia* flock within their impact assessments as a high priority.

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