The origin and future of an endangered crater lake endemic; phylogeography and ecology of *Oreochromis hunteri* and its invasive relatives

Florian N. Moser[1,2], Jacco C. van Rijssel[1,2,3], Benjamin Ngatunga[4], Salome Mwaiko[1,2], Ole Seehausen[1,2]

[1]Department of Aquatic Ecology, Institute of Ecology and Evolution, University of Bern, 3012 Bern, Switzerland
[2]Department of Fish Ecology & Evolution, EAWAG, Centre for Ecology, Evolution and Biogeochemistry, 6047 Kastanienbaum, Switzerland
[3]Wageningen Marine Research, Wageningen University & Research, IJmuiden, The Netherlands
[4]Tanzania Fisheries Research Institute, Box 9750, Dar es Salaam, Tanzania

Abstract

Cichlids of the genus *Oreochromis* ("Tilapias") are intensively used in aquaculture around the world. In many cases when "Tilapia" were introduced for economic reasons to catchments that were home to other, often endemic, *Oreochromis* species, the loss of native species followed. *Oreochromis hunteri* is an endemic species of Crater Lake Chala on the slopes of Mount Kilimanjaro, and is part of a small species flock in the upper Pangani drainage system of Tanzania. We identified three native and three invasive *Oreochromis* species in the region. Reconstructing their phylogeography we found that *O. hunteri* is closely related to, but distinct from the other members of the upper Pangani flock. However, we found a second, genetically and phenotypically distinct *Oreochromis* species in Lake Chala whose origin we cannot fully resolve. Our ecological and ecomorphological investigations revealed that the endemic *O. hunteri* is currently rare in the lake, outnumbered by each of three invasive cichlid species. It is mitochondrially, phenotypically and trophically distinct from all others. The occurrence of the formerly abundant *O. hunteri* in such small numbers, its narrow habitat restriction and its limited morphological variability suggest recent population decline and loss of niche breadth in this critically endangered endemic cichlid species.

Introduction

Biodiversity provides a multitude of ecosystem services (Watson et al. 2005) and for sustainable development, it is therefore essential to conserve biodiversity. The cichlids in the East African Lakes provide a biodiversity hotspot in freshwater ecosystems with several hundred endemic species in each of the big lakes Tanganyika, Malawi and Victoria (Genner et al. 2004) and many more endemic species in numerous smaller lakes (Wagner et al. 2014). While most of the species richness consists of the smaller haplochromine cichlids, most of the interest in fisheries and aquaculture is with the larger oreochromine cichlids, often referred to as "Tilapia". The oreochromine cichlids nonetheless made several small radiations, especially in some of the smaller lakes (Ford et al. 2015, Schliewen et al. 1994, Trewavas et al. 1972) that do not have haplochromines (Seehausen 2007). They also produced several endemic, often allopatric species in Eastern African rivers and small lakes (Trewavas 1983).

In tropical Africa, fish is one of the most important protein sources and in Eastern Africa millions of people depend heavily on fishery-related activities for their daily food or income (DeGraaf & Garibaldi 2014, FAO 2007, Ogutu-Ohwayo et al. 1997). As most East African aquatic ecosystems are dominated by cichlid fish, they provide important economic and food resources. Since the 1950s, the optimization of fishery yields led to the introduction of Nile Tilapia (*Oreochromis niloticus*, Linnaeus, 1758) in many places of Africa outside the species' distribution range, with negative impacts on the native *Oreochromis* species reported in many cases through competition and hybridization (Deines et al. 2014, Genner et al. 2013). *Oreochromis niloticus* is one of the world's most important species for food provisioning from aquaculture. It has been distributed for aquaculture not only within Africa, but worldwide (Casal 2006, Deines et al. 2016) and has established numerous wild populations in tropical and subtropical latitudes, including in China (Shuai et al. 2015), in the USA (Lowe et al. 2012), in India (Singh et al. 2013) and in Brazil (de Azevedo-Santos et al. 2011) to name only a few. Detrimental impacts on the local ecosystems due to resource competition with the native fish or habitat alterations due to the grazing behaviour, have been reported from various countries (Crutchfield 1995, Daga et al. 2016, Singh et al. 2013, Zhang et al. 2017). If introduced into catchments that have their own native

58 *Oreochromis* species, *O. niloticus* causes threats additionally through hybridization with the local

59 species (Angienda et al. 2011, D'Amato et al. 2007, Deines et al. 2014, Firmat et al. 2013).

60 In this study we investigate the status of a little known endemic crater lake species, *Oreochromis*

61 *hunteri* (Günther, 1889) of Lake Chala. We investigate aspects of its origins and distinctiveness within

62 the endemic species flock of the upper Pangani drainage, its distinctiveness versus several invasive

63 cichlids, and its ecology in the face of the presence of other cichlid species that have invaded the lake

64 in recent decades. *Oreochromis hunteri* is thought to be part of a small species flock of three or four

65 taxa in the upper Pangani drainage system. Lake Chala is a small (4.2 km2) but deep (90m) crater lake

66 with habitats of rock and lava sand. It is positioned at the eastern flank of Mount Kilimanjaro (3.317°S,

67 37.699°E) at 877 m above sea level, shared between Kenya and Tanzania. Inflow and outlet are both

68 subterranean and the water in the lake is very clear (Secchi depth ~6.6 m). Four different cichlid species

69 occur in Lake Chala: the endemic *Oreochromis hunteri*, an undescribed *Oreochromis* species

70 (sometimes erroneously referred to as *O. korogwe* (Lowe, 1955), here referred to as *O. sp.* "blue

71 head"), the widely distributed eastern red-breast tilapia *Coptodon rendalli* (Boulenger, 1897) and a

72 haplochromine cichlid of the riverine *Astatotilapia bloyeti* (Sauvage, 1883) complex. The latter three

73 most likely were introduced between 1951 and 1977 (Dadzie et al. 1988, Lowe 1955). Due to the co-

74 occurrence of an endemic cichlid species that had several thousand years to explore and exploit the

75 different ecological niches within Lake Chala, and one closely and two more distantly related species

76 that only arrived about 40 – 70 years ago, Lake Chala provides a system for investigating impacts of

77 introduced species on endemic cichlid communities of isolated lakes.

78 We reconstructed the phylogeography of the native and the recent colonizations, and investigated

79 ecology and ecomorphology of all four cichlid species in Lake Chala, as well as the oreochromine

80 cichlids of the upper Pangani/Ruvu drainage. We wanted to know if and how niches are partitioned

81 between the endemic and the invasive species to make predictions for the future of this unique little

82 ecosystem and its endemic cichlid species. We experimentally fished all habitats and several depth

83   gradients in Lake Chala, and combined studying habitat distribution of cichlids, analysis of 14

84   morphological traits and $\delta^{15}N$ and $\delta^{13}C$ stable isotopes.

85

Material and Methods

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Fish sampling

87

88   We sampled *Oreochromis* from four locations in North-Eastern Tanzania, in Lake Chala, in the Nyumba

89   ya Mungu Reservoir (dammed Pangani River), in the Ruvu River (the main inflow into the Nyumba ya

90   Mungu Reservoir) and in Lake Babati in November 2014 (figure 1). In Lake Chala gill nets with mesh

91   sizes from 12-22 mm were set at various depths in the benthic habitat in Lake Chala. Mesh sizes of the

92   nets were chosen to catch fish of comparable size of all species present in Lake Chala. This might have

93   led to a slight bias towards smaller individuals in the larger *Oreochromis* and *Coptodon* species. Nets

94   set below 47 m and nets set in the limnetic habitat (4 times three joined nets with 16, 19 and 22mm

95   mesh size) remained empty. Fish were carefully removed from the net and photographed from their

96   left side in a custom designed photo cuvette with a colour reference bar, a scale bar and a standardized

97   grey background. The fish was afterwards euthanized with an overdose of phenoxyethanol diluted in

98   lake water. From the right side of each fish, we removed the pectoral fin and fixed it in pure analytical

99   ethanol (100%) for DNA extractions, and we removed a piece of the epaxial muscle dorsal of the lateral

100   line, removed the skin, and dried the muscle tissue in an oven (60°C for 24 to 48 hours) for stable

101   isotope analysis. Whole fish were subsequently fixed in 4% formalin (buffered with borax) and were

102   later rinsed with water and stepwise transferred to 75% ethanol. Additionally, we bought several larger

103   individuals of *O. hunteri* from a local fisherman on the lake, who was fishing with hook and line. With

104   the same methods (gill netting) we caught and processed the fish from Lake Babati. In the Ruvu River,

105   the fish were caught with hook and line, and the fish from the Nyumba ya Mungu were bought from

106   the local fish market. Like this, we obtained samples from 4 sites and taxonomically identified 3 native

107   and 3 invasive *Oreochromis* species.

108

109   DNA extraction, sequencing and phylogenetic reconstruction

110   DNA was extracted from fin clips of *Oreochromis* from Lake Chala (18 *O. sp.* "blue head", 13 *O. hunteri*),

111   Nyumba ya Mungu (6 *O. jipe* (Lowe, 1955), 2 *O. esculentus* (Graham 1928), 1 *O. niloticus*), the Ruvu

112   River (14 *O. sp.* "blue head", 3 *O. pangani* (Lowe, 1955), 1 *O. niloticus*) and Lake Babati (2 *O. niloticus*,

113   4 *O. sp.*), and *Coptodon rendalli* from Lake Chala (N=4) and Nyumba ya Mungu (N=2). We used the

114   Qiagen DNeasy Blood&Tissue-Kit and sequenced 830 bp of the mitochondrial D-loop region.

115   We aligned our mitochondrial sequences in BioEdit v7.2.5 together with mitochondrial D-loop

116   sequences of most other *Oreochromis* species occurring in Eastern Tanzania from GenBank (D'Amato

117   et al. 2007, Kinaro et al. 2016, Nagl et al. 2001, Nyingi et al. 2009). A Templeton, Crandall and Sing (TCS)

118   network was built and visualized in PopART (Bandelt et al. 1999), to investigate and visualize the

119   relationship among the different populations and species.

120

121   Morphology and trophic ecology

122   For 27 *Oreochromis hunteri*, 74 *O. sp.* "blue head" and 10 *Coptodon rendalli* from Lake Chala, 20 *O.*

123   *jipe*, 5 *O. esculentus*, 5 *O. niloticus*, 2 *C. rendalli* and 10 *Astatotilapia* from the Nyumba ya Mungu

124   Reservoir, 6 *O. pangani*, 16 *O. sp.* "blue head" and 1 *O. niloticus* from the Ruvu River, we measured 15

125   linear morphometric distances that have proven powerful for quantifying taxonomically and

126   ecologically relevant morphometric variation in cichlids (Barel et al 1977, Witte 1984) using digital

127   callipers: standard length (SL), head length (HL), head width (HW), body depth (BD), lower jaw length

128   (LJL) and width (LJW), snout length (SnL), snout width (SnW), cheek depth (ChD), preorbital depth

129   (POD) and width (POW), interorbital width (IOW), eye length (EyL) and depth (EyD), and premaxillary

130   pedicel length (PPL). The same measurements for 274 Astatotilapia from Lake Chala were obtained

131   from Moser et al. (2018). All measurements were log-transformed and size-corrected by using the

132   standardized residuals of the pooled linear regression of each log-transformed trait against log-SL. The

133   largest *O. hunteri* that we bought from the fisherman at Lake Chala (SL > 19cm) were excluded as they

134   were four to eleven cm larger than the next smaller fish. All analyses, if not stated differently, were

135   performed in R 3.2.1 (R-Core-Team 2015).

136   To quantify morphological variation and differentiation, we performed principal component analyses.

137   To prevent that the PCA axes are mainly driven by the taxa with larger sample sizes, we used only ten

138   individuals of the species where sample sizes exceeded ten and all individuals for all other species for

139   the calculation of PCA axes. All other individuals were subsequently projected into the morphospace.

140   As an estimate of morphological differentiation, we calculated Bhattacharyya distances between the

141   different species in the PC1-PC2-morphospace and tested for significance with Hotelling's T-squared

142   test. To test for differences in morphological variation, we calculated morphospace occupation for

143   each species. For this purpose, we subsampled 1000 times ten individuals per species and calculated

144   the morphospace occupation using the R-function chull. We report the mean of these 1000

145   permutations and used the permutations to assess whether morphospace occupation is significantly

146   different between species. To investigate which traits are most distinct between *O. hunteri, O. sp.*

147   "blue head", *O. jipe* and *O. pangani*, we additionally performed pairwise linear discriminant analyses

148   (LDA).

149   For all fish from Lake Chala, we performed stable isotope analyses. Dried muscle tissue was ground

150   into fine powder using a QIAGEN Tissuelyzer II with five-millimetre stainless steel beads (QIAGEN). Of

151   this powder, 0.25 - 0.35 mg was used to analyse δ^{13}C and δ^{15}N values in a ThermoFisher Scientific Flash

152   2000 elemental analyser coupled through a Conflo IV interphase to a Delta V Advantage Isotope Ratio

153   Mass Spectrometer (IRMS). To quantify ecological variation and differentiation, we calculated

154   Bhattacharyya distances between the different species based on the stable isotope ratios and

155   tested for significance with Hotelling's T-squared test.

156

157   Results

158   Taxonomy, diversity and phylogeography of *Oreochromis* in Lake Chala and the nearby

159   Ruvu/Pangani system

160　　We found evidence for three native (*Oreochromis hunteri, O. jipe* and *O. pangani*) and three non-native

161　　(*O. niloticus, O. esculentus* and *O. sp*. "blue head") oreochromine species in the Pangani/Ruvu drainage,

162　　including Lake Chala (figure 2). The D-loop haplotype network revealed that *O. hunteri* is most closely

163　　related to the geographically nearby Pangani-system endemics, *O. jipe* and *O. pangani*, but does not

164　　share any haplotypes with either of these (figure 3). This confirms earlier morphology-based

165　　suggestions, that these three species form a "Pangani system flock" of closely related species

166　　(Trewavas 1983). We did not encounter any *Oreochromis hunteri* outside Lake Chala, neither did we

167　　encounter either of the other native Ruvu/Pangani taxa in Lake Chala.

168　　However, we found a second, genetically distinct *Oreochromis* species in Lake Chala whose origin we

169　　could not fully resolve. This species, which we refer to as *O. sp.* "blue head", does not belong to the

170　　Ruvu/Pangani radiation in its mitochondrial lineage but is more closely related to *O. urolepis* from the

171　　Tanzanian East Coast rivers (figure 3) and also shared the distinctive large midlateral blotches with the

172　　latter (figure 2). In *O. sp.* "blue head" we found two very distinct haplotype groups that differ from

173　　each other by 12 mutations (in 347 bp), suggesting that the population may be a mix of introductions

174　　from two distinct source populations. We identified one individual from Lake Chala phenotypically as

175　　*O. niloticus* or an *O. niloticus*-hybrid but its mitochondrial haplotype was one of the two *O. sp.* "blue

176　　head" haplotypes. We also found two non-oreochromine cichlids in Lake Chala, *Coptodon rendalli* and

177　　*Astatotilapia cf. bloyeti*. Both also occur in the Ruvu/Pangani system, where *Astatotilapia cf. bloyeti* is

178　　native and *C. rendalli* has been introduced.

179　　In the Ruvu River we found matching phenotypic and genetic evidence for the presence of three

180　　*Oreochromis* species: the native *O. pangani*, the introduced *O. niloticus* and the unidentified *O. urolepis*

181　　clade taxon that we also found in Lake Chala (*O. sp.* "blue head", both haplotypes). In the Nyumba ya

182　　Mungu Reservoir finally, we found matching phenotypic and genetic evidence for presence of three

183　　*Oreochromis* species too, but here these were *Oreochromis jipe* (supposedly introduced from Lake Jipe

184　　further upstream in the catchment) and introduced *O. niloticus* and *O. esculentus*.

185   In Lake Babati we found phenotypic and genetic evidence for the presence of *O. niloticus*. Further we

186   found mitochondrial haplotypes belonging to *O. spilurus* and *O. esculentus* (figure 3)*,* but specimens of

187   these two haplogroups could not be distinguished from each other phenotypically.

188

189   Ecological and phenotypic species differentiation and niche partitioning

190   We find that in Lake Chala each of the invasive *O. sp* "blue head", *C. rendalli* and *A. cf. bloyeti* are more

191   abundant than the native *O. hunteri* (figure 4). With the exception of a few fish caught shallower, *O.*

192   *hunteri* seems to be restricted to a depth range between 20 and 45m, i.e. the deeper half of the

193   oxygenated habitat. Even at those depths, it was outnumbered between 3 and 20 times by other cichlid

194   species (figure 4). However, our stable isotope data suggest significant feeding niche partitioning

195   between *O. hunteri* and all other species (figure 5). In fact, all four species in Lake Chala differ

196   significantly from each other in their stable isotope signatures (figure 5, table 1). *O. hunteri* feeds on

197   slightly more depleted carbon sources than *O.* sp. "blue head" and *C. rendalli* but fully overlapping with

198   *A. cf. bloyeti*. On the other hand, *O. hunteri* feeds at a slightly higher trophic level than *O.* sp. "blue

199   head" and *C. rendalli* and lower than *A. cf. bloyeti*. The latter species feeds predominantly on

200   invertebrates (insect larvae, zooplankton) and small fish (Moser et al. 2018) and its mean δ^{15}N value is

201   10.6. *Coptodon rendalli* is known as a macrophyte eater elsewhere but probably grazes mostly on dead

202   wood and organic debris in Lake Chala where macrophytes are nearly absent. Its mean δ^{15}N-value in

203   Lake Chala is about 8.0, slightly higher than *O. sp*. 'blue head' with a mean of 7.7, and nearly one trophic

204   level below *Astatotilapia*, as expected if it feeds mainly on plant matter. The mean δ^{15}N-value of *O.*

205   *hunteri* in our data is about 8.4, with the largest fish slightly but not much higher. This suggests that *O.*

206   *hunteri*, despite no evidence of a unique habitat, may have a unique feeding niche that includes both

207   vegetable and animal matter.

208   Our morphological analysis reveals that *O. hunteri* is clearly distinct eco-morphologically from all other

209   species in the system too. Within Lake Chala all four species are morphologically distinct from each

210   other (table 1), but the strongest overlap in morphospace is between *Oreochromis hunteri* and the

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211   invasive *Oreochromis* sp. "blue head" (figure 6a). The main difference between these species are a

212   longer head, a less deep body and a narrower snout in *O. hunteri* compared to *O. sp*. "blue head" (table

213   2). A corresponding pattern is detected outside Lake Chala, where all species except *Oreochromis jipe*

214   and *O. pangani* can be morphologically distinguished in the PCA (table 3, figure 6b) with the highest

215   PC1 and PC2 values for the native *Oreochromis* species (*O. pangani* and *O. jipe*). Whereas PC1 (-ChD

216   and –LJL) mainly separates *Oreochromis* (small cheek depth and short lower jaws) from *Astatotilapia*

217   and *Coptodon*, PC2 (-IOW, -POD, -SnW, -BD) separates the Pangani *Oreochromis* (*O. hunteri*, *O. jipe*

218   and *O. pangani* with comparably narrow interorbital width and snout width, and low body depth) from

219   the introduced *Oreochromis* and *Coptodon* (comparably wide interorbital width and snout width, and

220   deep body depth) from *Astatotilapia*. Together, these two axes explain 74.5% of the overall variation

221   (45.7% and 28.7% respectively).

222   Within the endemic Pangani species flock, *O. hunteri* is morphologically distinct from both *O. jipe* and

223   *O. pangani* (with a longer and wider head, shorter lower jaws and smaller eyes in *O. hunteri*; table 2),

224   whereas we could not detect significant differentiation between the latter two (table 4, figure 6c). We

225   notice that whereas in the Pangani River (Nyumba ya Mungu Reservoir), the native species (*O. jipe*)

226   occupies significantly more of the morphospace than the newly arrived *O.* "blue head", the latter

227   occupies more (though not significantly more) of the morphospace than *O. hunteri* in Lake Chala (table

228   5, figure 6a and 6b).

229

Discussion

231   *Oreochromis hunteri* and the oreochromine cichlids of the Pangani/Ruvu drainage

232   *Oreochromis hunteri* Günther 1889 is one of the least known African cichlids, despite it being the type

233   species of the genus that contains the world's most important aquaculture fish. When Lowe (1955)

234   described a small flock of *Oreochromis* species endemic to the upper Pangani River drainage of

235   Tanzania, she was not sure if *O. hunteri* belonged to this species flock. Trewavas (1983) suggested,

236   based on morphological grounds, that *O. hunteri* belonged to the Pangani flock and that it was closely

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237   related to *O. jipe.* Trewavas characterized the members of the flock by high numbers of vertebrae,

238   scales in the lateral line and dorsal fin rays, and suggested that four species belonged to it: *O. pangani*

239   of rivers Ruvu and Pangani, *O. girigan* of Lake Jipe, *O. jipe* of Lake Jipe and possibly the Ruvu/Pangani

240   River, and *O. hunteri* of Lake Chala. This little flock had not been studied since, and never in a

241   phylogeographic context until recently (Dieleman et al. 2018)

242   We collected three endemic and three invasive *Oreochromis* species in the upper Pangani drainage

243   including Lake Chala. We find strong support for the proposed monophyly of the upper Pangani flock

244   in the mitochondrial lineage (figure 3), confirming the results of Dieleman et al. (2018), who sequenced

245   two mitochondrial markers. Their proximity in morphospace further supports their close relationship

246   (figure 6c). Among the three endemic species that we obtained, *O. hunteri* is genetically and

247   phenotypically distinct and indeed restricted to Lake Chala. *O. jipe* appears to be common in the

248   Nyumba ya Mungu Reservoir. This was already reported by Trewavas (1983) but it has never been

249   completely clear whether its original range comprised of the Pangani/Ruvu River and Lake Jipe or was

250   confined to Lake Jipe (Lowe 1955, Trewavas 1983). Finally, we found *O. pangani* in the Ruvu River, the

251   main inlet to the reservoir, but we did not find *O. jipe* there. We cannot rule out that some *O. pangani*

252   where among our samples from Nyumba ya Mungu, but all our larger individuals had 4 anal fin spines,

253   a defining trait of this species according to Lowe (1955) and Trewavas (1983), and the typical coloration

254   of *O. jipe*, whereas all our Ruvu River samples had 3 anal spines (Lowe 1955, Trewavas 1983).

255   Beside the introduced *O. niloticus* that we found in the Ruvu River and the reservoir, and *O. esculentus*

256   which we found in the reservoir and that were already reported in the Ruvu/Pangani by Lowe (1955),

257   we found a third most likely non-native oreochromine cichlid species in the system that we refer to as

258   *O. sp.* "blue head". *O. sp.* "blue head" occurs in two very divergent mitochondrial haplotypes. Both

259   were found in Lake Chala as well as in the Ruvu River. One of these two haplotypes suggests, identity

260   with or a close relationship to *O. urolepis urolepis* (figure 3). As the second haplotype is divergent by

261   twelve mutations and not closely related to any known *Oreochromis* species, the origin and the

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262   evolutionary relationship of *O. sp*. "blue head" remain partly unresolved but it is likely that it emerged

263   from admixture of two divergent lineages in the *O. urolepis* clade.

264

265   The origin of *Oreochromis hunteri*

266   *O. hunteri* appears to have evolved a more limnetic life style when compared to typical *Oreochromis*

267   including its close allopatric relatives *O. jipe* and *O. pangani*, but also the distant relative *O.* sp. "blue

268   head" with which it is now sympatric in Lake Chala. This is apparent both from its body shape and

269   stable isotopic signatures. *O. hunteri* evolved in isolation in the crater lake for at least 19'000 years

270   (Dieleman et al. 2015). Cichlids are known for undergoing sympatric speciation into distinct ecomorphs

271   with different habitat and/or feeding adaptations. This has involved divergence into limnetic and

272   benthic forms in several oreochromine cichlids, and divergence into shallow and deep benthic forms

273   in several haplochromine cichlids. There is no evidence of such divergence within the contemporary

274   population of *O. hunteri*. Analyses of fossil teeth from Lake Chala sediment cores, and comparison with

275   contemporary teeth revealed that *O. hunteri* and *O. sp*. "blue head" together show higher

276   morphological disparity in tooth shape than the fossil assemblage. However, the fossil assemblage

277   occupies a larger volume in morphospace than the contemporary *O. hunteri* alone, with some fossil

278   teeth overlapping in morphospace with *O. sp*. "blue head" (Dieleman et al. 2015). This might indicate

279   that *O. hunteri* was ecologically more diverse historically than it is now. Especially more robust teeth

280   that may facilitate scraping of benthic algae are absent in the contemporary *O. hunteri* but dominate

281   the introduced species. To conclude whether *O. hunteri* has been more diverse in the past or whether

282   there was perhaps a second more littoral native species, more fossil data would be required.

283

284   The future of *Oreochromis hunteri*

285   Our data suggest major risks for the persistence of *O. hunteri* arising from the recent introductions of

286   four other cichlid species to Lake Chala. While currently very rare in the lake, and perhaps only present

287   as hybrids, the potential presence of *O. niloticus* is worrying given its invasiveness across large parts of

the world and its known impact on native *Oreochromis* elsewhere through genetic introgression

coupled with competitive advantages (Angienda et al. 2011, Deines et al. 2014). More immediate

impacts on *O. hunteri* are likely to come from the presence of the three other introduced cichlid

species, all of which are more abundant in Lake Chala now than is *O. hunteri*. Our data suggest massive

habitat overlap with all three species, and dietary overlap mainly with *O.* sp. "blue head". The latter

species is probably able to hybridize with *O. hunteri* (as probably is *O. niloticus*) whereas *C. rendalli* and

A. cf. bloyeti most likely are too distantly related to *O. hunteri* to hybridize. Our stable isotope data,

our and recently published (Dieleman et al. 2018) morphological data and published tooth shape data

(Dieleman et al. 2015) all suggest that *O. hunteri* is phenotypically and ecologically distinct from *O.* sp.

"blue head". Although we had a few fish in our data that we could not clearly identify, we did not

detect mismatches between phenotype and mitochondrial haplotype in the individuals that we

identified with certainty. However, the extent of introgression and associated threats to the

persistence of *O. hunteri* need now to be analysed using microsatellite DNA or next generation

sequencing.

The complementarity in stable isotope signatures between *O. hunteri* and the invasive species in Lake

Chala is remarkable. We do not know if the current feeding and habitat niche of *O. hunteri* is its original

range, or if its niche has shrunk or shifted in response to the arrival of the invasive species. However,

several observations point to possible recent change:

First, in 1951 Lowe observed shoals of young feeding on algae and debris from the bottom between

the rocks near the shore (Lowe 1955). We have not seen any *O. hunteri* in the shallow waters despite

many diving hours, but we encountered all three invasive species very abundantly. In 1995, one of us

(OS) photographed a pelagic shoal of subadult *O. hunteri* several meters above the ground (figure 7).

We have not seen any such shoals in 2014.

Second, the *Oreochromis* teeth in the fossil assemblage studied by Dieleman et al (2015) occupied a

larger volume in morphospace than the contemporary *O. hunteri* teeth, with some fossil teeth

overlapping in morphospace with the invasive *O. sp.* 'blue head.

314 Third, the relative morphospace occupation between the introduced and the native species is much

315 less favourable to the native *Oreochromis hunteri* in Lake Chala than for the native species *O. jipe* in

316 the Nyumba ya Mungu Reservoir. Given that *O. hunteri* had several thousand years of evolution in Lake

317 Chala (Dieleman et al. 2015), and therefore the possibility to adapt to the different niches within the

318 lake without competing with any other fish species, we would have expected *O. hunteri* to be

319 phenotypically more variable than the invasive species. This is indeed what we see in *O. jipe* compared

320 to *O. sp.* "blue head". However, we see the opposite in Lake Chala where the invasive *O. sp.* "blue

321 head" is phenotypically significantly more diverse than *O. hunteri*. This again might indicate, that *O. sp.*

322 "blue head" displaced *O. hunteri* from parts of its morphospace and likely also its ecological niche.

323

Conclusions

325 We provide evidence for the presence of three endemic and three invasive *Oreochromis* species in the

326 upper Pangani drainage (Nyumba ya Mungu Reservoir, Ruvu River and Lake Chala). The three native

327 species that we collected in the Pangani/Ruvu and in Lake Chala have retained their phenotypic and

328 mitochondrial distinctiveness despite their current sympatry with several non-native species that

329 arrived since the 1950s in the Ruvu/Pangani and since the 1970s in Lake Chala. However, there is

330 serious concern for the future persistence at least of the endemic *O. hunteri* which is currently the

331 least abundant cichlid species in Lake Chala, phenotypically less diverse than its allopatric sister species

332 and the introduced sympatric species in Lake Chala, and missing from habitats where it was historically

333 recorded. Nuclear genetic marker studies are required to investigate potential introgressive

334 hybridization with the numerically dominant invasive species.

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Table 1: Differentiation between the different species within Lake Chala in morphology (lower left) and trophic ecology (upper right), measured as the Bhattacharyya-distance in the morphospace spanned by PC1 and PC2 and the stable isotope space respectively. Significant differentiation is indicated by asterisks (*: p<0.05, **: p<0.01).

	Astatotilapia	*O. sp.* "blue head"	*O. hunteri*	*C. rendalli*
Astatotilapia		3.79**	1.98**	1.59**
O. sp. "blue head"	4.98**		0.28**	0.36**
O. hunteri	12.90**	0.90**		0.44**
C. rendalli	1.56**	4.97**	21.34**	

Table 2: Coefficients for the morphological traits in the pairwise LDA. Coefficients higher than 10 or lower than -10 are highlighted bold (HUN = *O. hunteri*, BLU = *O. sp*. "blue head", JIP = *O. jipe*, PAN = *O. pangani*). The last row indicates which species has the more positive LD1.

	HUN vs BLU	HUN vs JIP	HUN vs PAN	PAN vs JIP
Head length	**22.6**	**38.1**	**40.2**	**24.9**
Head width	5.4	**17.8**	7.9	5.1
Body depth	**-10.5**	-9.8	**29**	-7.6
Lower jaw length	3	2.4	9.6	1.6
Lower jaw width	1.7	-1.0	-2.1	5.5
Snout length	-3.5	-9.4	**-23.3**	**-10.3**
Snout width	**-19.6**	-2.9	**-44.1**	**47.6**
Cheek depth	-3.5	1.6	0.2	3.9
Preorbital depth	5.4	4.7	**11.3**	0.9
Interorbital width	-0.6	-9.6	**-11.7**	**-35.3**
Eye length	1.9	**-11.8**	5.5	**-19.2**
Eye depth	-0.1	**-14.8**	**-28**	6.4
Preorbital width	**-14.1**	**-11.9**	-0.9	**-19.2**
Positive LD1	HUN	HUN	HUN	PAN

Table 3: Morphological differentiation between the different sampled *Oreochromis* species in the Nyumba ya Mungu Reservoir and its inflow, the Ruvu River, measured as the Bhattacharyya-distance in the morphospace spanned by PC1 and PC2. Significant differentiation is indicated by asterisks (*: p<0.05, **: p<0.01).

	Astatotilapia	*O. sp.* "blue head"	*O. jipe*	*O. pangani*	*O. esculentus*
Astatotilapia					
O. sp. "blue head"	14.37**				
O. jipe	18.96**	2.63**			
O. pangani	16.44**	3.13**	0.59		
O. esculentus	53.94**	16.26**	2.93**	11.11**	
O. niloticus	18.98**	2.11**	1.20**	5.46**	3.54**

378 **Table 4:** Differentiation between the different endemic species of the Pangani/Ruvu drainage including
379 Lake Chala in morphology, measured as the Bhattacharyya-distance in the morphospace spanned by
380 PC1 and PC2. Significant differentiation is indicated by asterisks (*: $p < 0.05$, **: $p < 0.01$).

	O. hunteri	*O. jipe*
O. hunteri		
O. jipe	0.76**	
O. pangani	0.98*	0.59

381
382
383
384

385 **Table 5:** Morphospace occupation after correcting for differences in sample sizes for the three
386 focal species of this study and p-values for the difference in morphospace occupation between
387 species.

Morphospace occupation		*O. hunteri*	*O. sp.* "blue head" (Chala)	*O. jipe*
O. hunteri	2.19			
O. sp. **"blue head" (Chala)**	3.23	> 0.20		
O. jipe	2.32	> 0.20	> 0.20	
O. sp. **"blue head" (Ruvu)**	0.80	< 0.01	< 0.01	< 0.01

388

Fig. 1 Map of the upper Pangani River drainage with the sampling sites used in this study: Lake Chala, the Pangani

Reservoir Nyumba ya Mungu and its main inlet, the Ruvu River, and Lake Babati

.

Fig. 2 The oreochromine cichlid species of the upper Pangani system. From left to right and top to bottom: *Oreochromis hunteri* from Lake Chala, adult male and subadult male; *O. jipe* from Nyumba ya Mungo, two adult males; *O. pangani* from the Ruvu River, two subadults; *O. sp*. "blue head" from Lake Chala, adult male with enlarged jaws; adult individual without enlarged jaws; *O. niloticus* from the Ruvu River (subadult); *O. sp.* adult male from Lake Babati.

Fig. 3 The phylogeographic relationships of the *Oreochromis* spp of Lake Chala, the upper Pangani and Lake Babati. TCS haplotype network of 347 bp of mitochondrial D-loop. We only used 347 bp because most sequences used from GenBank were restricted to this shorter fragment. The size of the circles indicates the number of individuals with a given haplotype in our data, ticks between the circles indicate single point mutations. Dark blue are individuals collected from Lake Chala, black individuals collected from the Nyumba ya Mungu Reservoir, green individuals collected from the Ruvu River (upper Pangani, inflow to the Nyumba ya Mungu Reservoir), turquoise individuals collected in Lake Babati, brown individuals were taken from GenBank (Nagl et al 2001, D'Amato et al 2007, Nyingi et al 2009, Kinaro et al 2016). Whereas *O. hunteri* is very closely related to the upper Pangani endemics *O. pangani* and *O. jipe*, we observed two only distantly related haplotype groups within *O. sp* "blue head", the other *Oreochromis* from Lake Chala and the Ruvu River. One of these is shared with the Rufiji-Tilapia (*O. urolepis urolepis*).

Fig. 4 Relative abundance of the different cichlid species at different water depths in Lake Chala. All four cichlid species were present at all three geographical sampling locations. All species occurred from the very shallow areas down to a water depth of at least 40 m but none was found deeper than 47 m. *Astatotilapia* were the most abundant taxon in the shallow habitat, whereas *Coptodon rendalli* was most abundant in the deeper habitats. The two *Oreochromis* species were both caught in the deep and in the shallow habitat, but were more common

418   in the deeper habitat and virtually absent from intermediate water depths. The endemic *Oreochromis hunteri*

419   was clearly the least abundant species in our catches. Abundance data is probably slightly biased towards the

420   smaller *Astatotilapia* and subadult *Oreochromis* and *Coptodon*, because we used a maximal mesh size of 22 mm

421   and therefore likely missed very large individuals of *Oreochromis* and *Coptodon*. Sample sizes are given to the

422   right of each depth category.

423

424

425   **Fig. 5** Ecological variation and differentiation among the four cichlid species in Lake Chala represented by the

426   stable isotope plot of the four species: the endemic *Oreochromis hunteri* (green), *O. sp*. "blue head" (blue),

427   *Coptodon rendalli* (black) and *Astatotilapia cf. bloyeti* (red). The size of the symbols corresponds to the size of

428   each specimen.

429

430

431   **Fig. 6** Morphological variation and differentiation among the cichlid species in Lake Chala and the Pangani

432   drainage. a) PC-plot on linear morphological traits with all individuals from Lake Chala (triangles) highlighted. (b)

433   Same morphospace with all fish from the Ruvu River (diamonds) and the Nyumba ya Mungu Reservoir (dots)

434   highlighted (b). (c) Morphospace occupation of the endemic species of the Pangani drainage and Lake Chala *O.*

435   *jipe* (yellow) from the Nyumba ya Mungu, *O. pangani* (orange) from the Ruvu River and *O. hunteri* from Lake

436   Chala (green) and (d) morphospace occupation of the introduced *Oreochromis sp.* "blue head" in Lake Chala

437   (blue) and in the Ruvu River (lightblue). The colour of the dots represent the different species with *Astatotilapia*

438   *cf. bloyeti* in red, *O. hunteri* in green, *O. sp*. 'blue 'head' in blue, *C. rendalli* in black, *O. pangani* in orange, *O. jipe*

439   in yellow, *O. niloticus* in purple and *O. esculentus* in brown. The symbols of the dots represents the catching

440   location with triangle for Lake Chala, dots for the Nyumba ya Mungu Reservoir and diamonds for the Ruvu River.

441

442

443   **Fig. 7** Underwater picture of Lake Chala. (a) Picture from 2014 of all three invasive species at 6m water depth.

444   Note the high density of *Astatotilapia cf. bloyeti* (red) and *Coptodon rendalli* (black) in this habitat. (b) A school

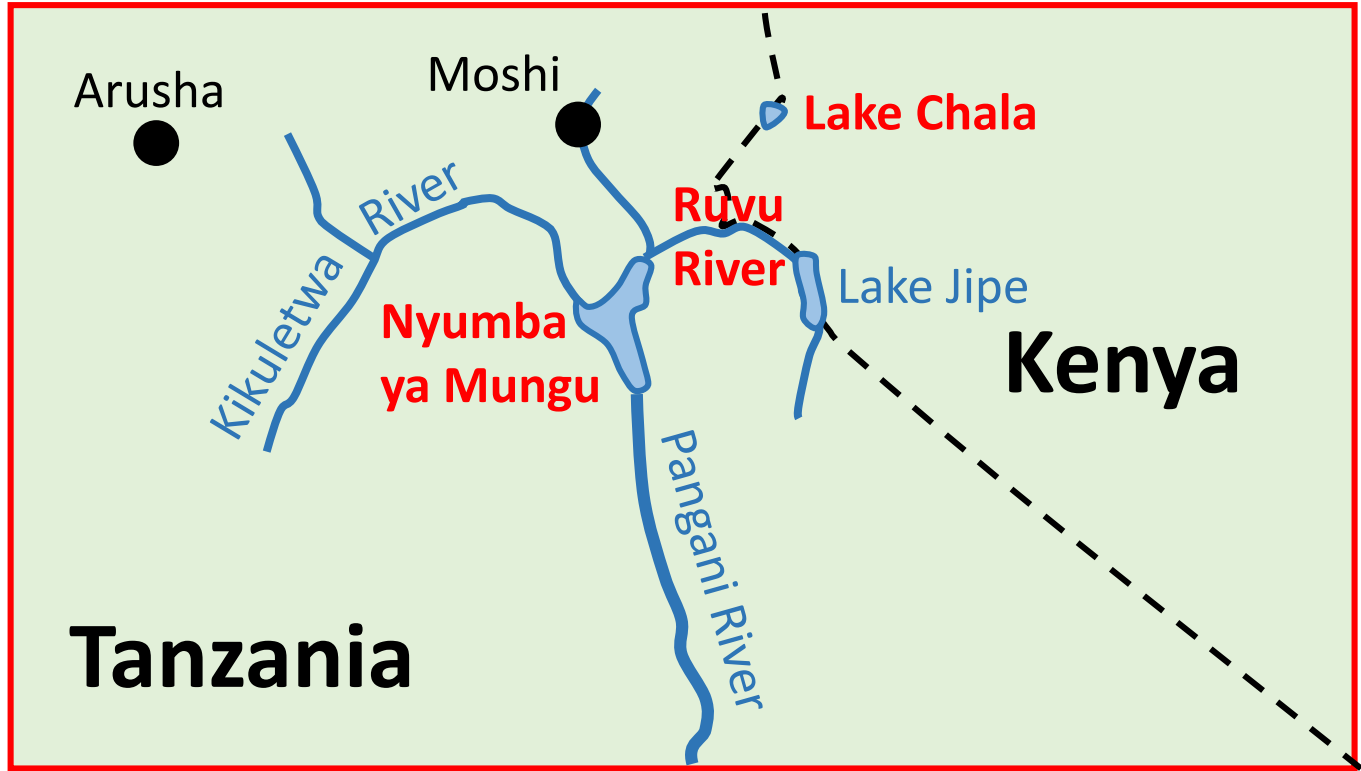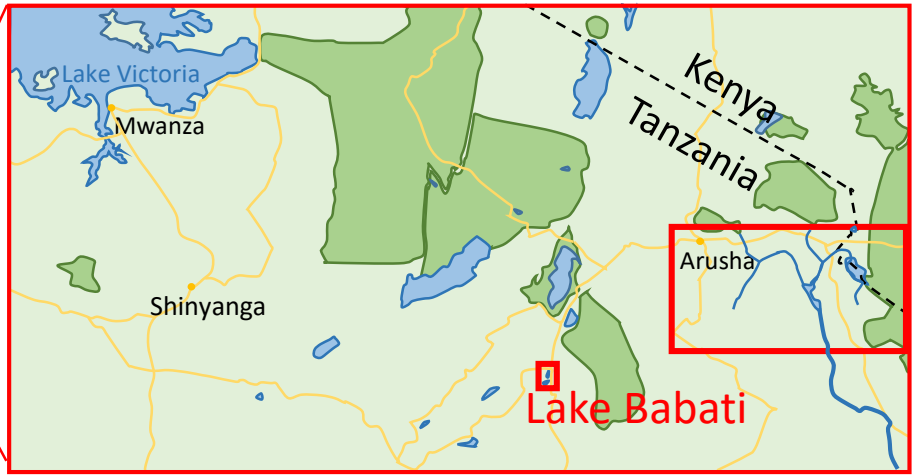445   of *O. hunteri* swimming in the open water in 1995.

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535

Figure 1

Figure 1

Figure 2

O. hunteri adult

O. hunteri subadult

O. jipe adult

O. jipe adult

O. pangani subadult

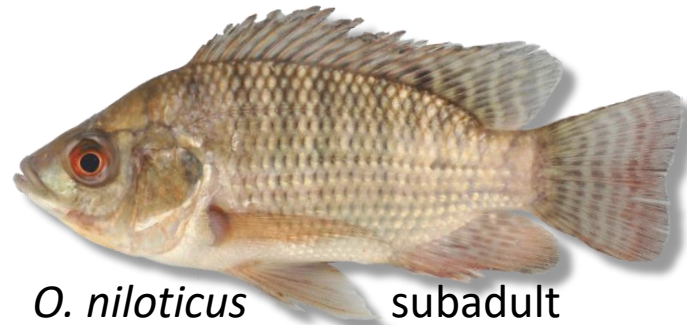*O. pangani* subadult

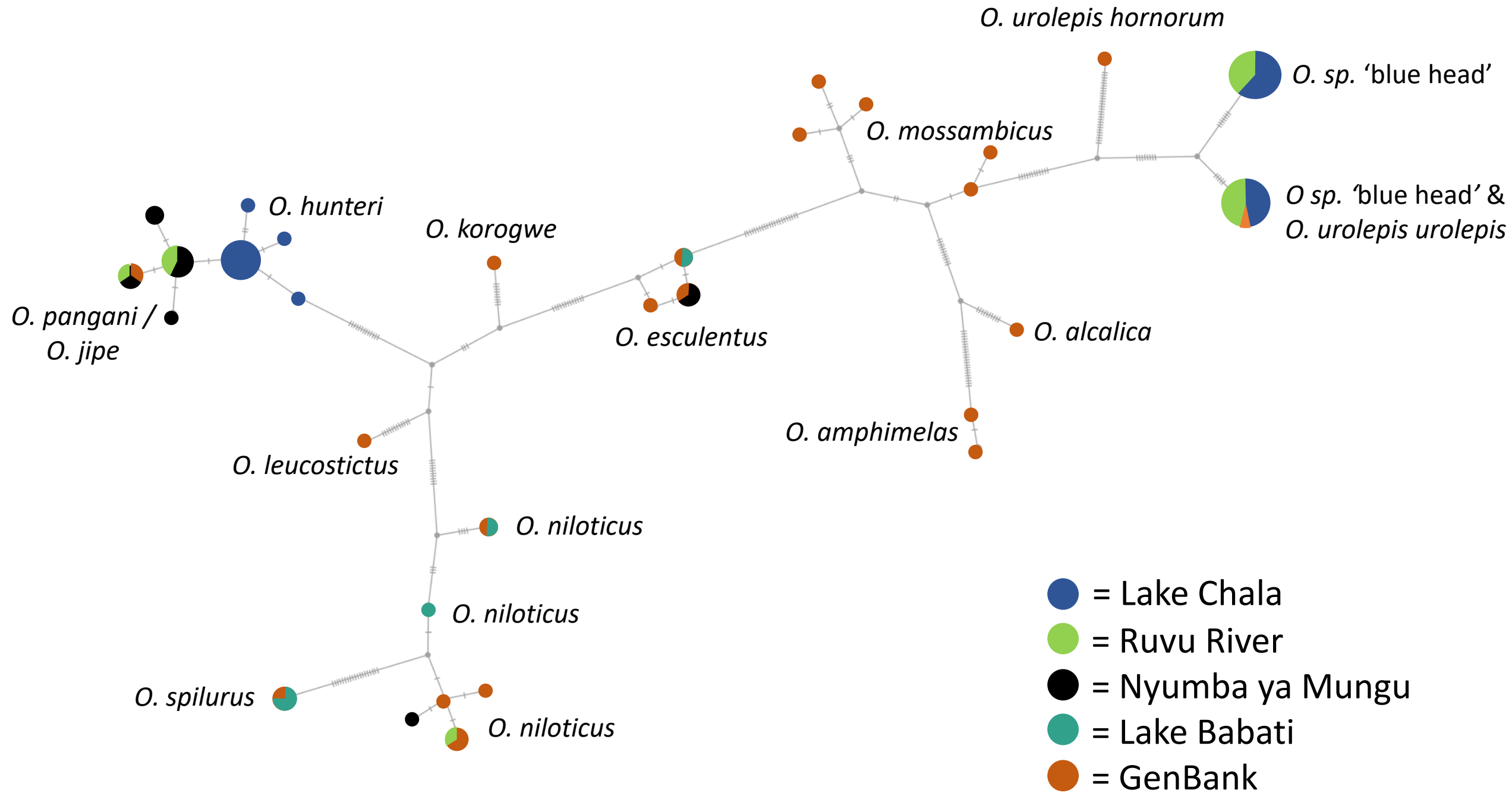*O. sp.* «blue head» adult

O. sp. «blue head» adult

O. niloticus subadult

O. sp. «Babati» adult

Figure 3

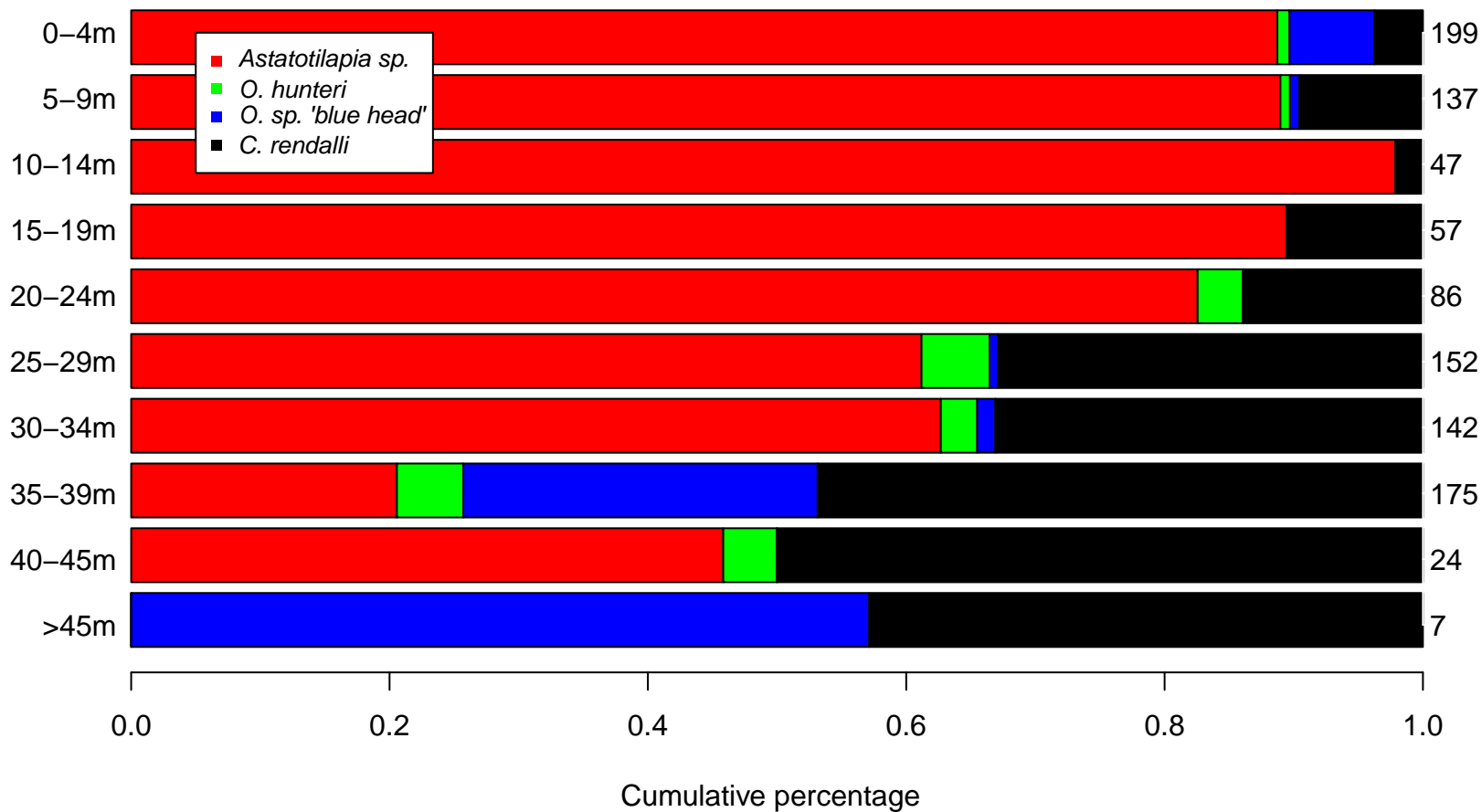Click here to access/download;Figure;Fig3.pdf ⬇

Figure 3

Figure 4

Species abundance in Lake Chala

Figure 5

Figure 6

a) b) c) d)

Legend:
- Astatotilapia (red)
- O. hunteri (green)
- O. blue head (blue)
- C. rendalli (black)
- O. pangani (orange)
- O. jipe (yellow)
- O. niloticus (purple)
- O. esculentus (dark red)
- Chala (grey triangle)
- Nyumba (grey circle)
- Ruvu (grey diamond)

Figure 7

a) 2014

O. sp. «blue head»

A. cf. bloyeti

C. rendalli

b) 1995