Whole-genome sequences of Malawi cichlids reveal multiple radiations interconnected by gene flow

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The hundreds of cichlid fish species in Lake Malawi constitute the most extensive recent vertebrate adaptive radiation. Here we characterize its genomic diversity by sequencing 134 individuals covering 73 species across all major lineages. The average sequence divergence between species pairs is only 0.1-0.25%. These divergence values overlap diversity within species, with 82% of heterozygosity shared between species. Phylogenetic analyses suggest that diversification initially proceeded by serial branching from a generalist Astatotilapia-like ancestor. However, no single species tree adequately represents all species relationships, with evidence for substantial gene flow at multiple times. Common signatures of selection on visual and oxygen transport genes shared by distantly related deep-water species point to both adaptive introgression and independent selection. These findings enhance our understanding of genomic processes underlying rapid species diversification, and provide a platform for future genetic analysis of the Malawi radiation.
Within the Lake Malawi radiation are genetically closely related species. Diplotaxodon benthic groups show levels of diversity that are comparable to those of the mbuna and shallow benthic (Fig. 1). Despite their extensive phenotypic differentiation, species with higher than the divergence between two distinct species. The average of 2.0 × 10⁻³ per bp. The maximum of the fixation index and of the expected and previously observed high levels of incomplete lineage sorting (ILS)²⁵. Supplementary Fig. 2 shows values of and of the fixation index for comparisons between the seven eco-morphological groups and Supplementary Fig. 3 shows patterns of linkage disequilibrium across the radiation, within and between species.

**Low per-generation mutation rate.** It has been suggested that the species richness and morphological diversity of teleosts in general and of cichlids in particular might be explained by elevated mutation rates compared to other vertebrates²⁶. To obtain a direct estimate of the per-generation mutation rate, we reared offspring of...
three species from three different Lake Malawi groups (A. calliptera, Aulonocara staughtrani and Lethrinops leothyrum). We sequenced both parents and one offspring of each to high coverage (40X), applied stringent quality filtering, and counted variants present in each offspring but absent in both its parents (Supplementary Fig. 4). There was no evidence for significant difference in mutation rates between species. The overall mutation rate ($\mu$) was estimated at 3.5 x 10^{-9} (95% confidence interval (CI): 1.6 x 10^{-9} to 4.6 x 10^{-9}) per bp per generation, approximately three to four times lower than in humans^7, although, given much shorter mean generation times, the per-year rate is still expected to be higher in cichlids than in humans. We note that ref. 26 obtained a much higher mutation rate estimate (6.6 x 10^{-8} per bp per generation) in Midas cichlids, but from relatively low-depth sequencing of restriction-site-associated markers that may have made accurate verification more difficult. We also note that our per-generation rate estimate, although low, is still higher than the lowest $\mu$ estimate in vertebrates: 2 x 10^{-9} per bp per generation recently reported for Atlantic herring^72. By combining our mutation rate with nucleotide diversity ($\pi$) values, we estimate the long term effective population sizes ($N_e$) to be in the range of approximately 50,000 to 130,000 breeding individuals (with $N_e = \pi/4\mu$).

**Genome data support for eco-morphological groupings.** PCA of the whole-genome genotype data generally separates the major eco-morphological groups (Fig. 1c). The most notable exceptions to this are (1) the utaka, for which some species cluster more closely with deep benthics and others with shallow benthics, and (2) two species of the genus Aulonacara, A. staughtrani and A. stевичи, which are located between the shallow and deep benthic groups. Although these have enlarged lateral-line sensory apparatus like many deep benthic species including other Aulonacara, they are typically found in shallower water^23. Another interesting pattern in the PCA plot is that the utaka and benthic samples are often spread along principal component (PC) axes (Fig. 1c, Supplementary Fig. 5), a pattern typical for admixed populations (for example ref. 26). Along the two main PCs, the deeper-water benthic species extend towards the deep-water Diplotaxodon, an observation we will return to in the context of gene flow and shared mechanisms of depth adaptation.

To further verify the consistency of group assignments, we tested whether pairs of species from the same group always share more derived alleles with each other than with any species from other groups. Group assignments were again supported, except for the four species also highlighted in the PCA: the two shallow-living Aulonacara are closer to shallow benthics than to deep benthics in 71% and 82% of tests respectively when comparing these alternatives, and Copadichromis trimaculatus is closer to shallow benthics than to utaka in 58% of the comparisons. Copadichromis cf. trewava-sae always clustered with shallow benthics; therefore, we treat it as a member of the shallow benthic group henceforth. With the three intermediate samples removed and C. cf. trewava-sae realligned, all other species showed 100% consistency with their group assignment.

**Allele sharing inconsistent with tree-like relationships.** The above observations suggest that some species may be genetically intermediate between well defined groups, consistent with previous studies that have suggested that hybridization and introgression subsequent to initial separation of species may have played a significant part in cichlid radiations, including in lakes Tanganyika^13,14,16 and Malawi^18,20. Where this happens, there is no single tree relating the species.

To assess the overall extent of violation of tree-like species relationships, we calculated Patterson’s D statistic (the ABBA-BABA test)^32,33 for all possible trios of Lake Malawi species, without assuming any a priori knowledge of their relationships. N. brichardi from Lake Tanganyika was always used as the outgroup. The test statistic $D_{\text{min}}$ is the minimum absolute value of Patterson’s $D$ for each trio, across all possible tree topologies. Therefore, a significantly positive $D_{\text{min}}$ score signifies that the sharing of derived alleles between the three species is inconsistent with a single species tree relating them, even in the presence of incomplete lineage sorting.

Overall, 62% of trios (75,616 out of 121,485) have a significantly positive $D_{\text{min}}$ score (Holm–Bonferroni FWER < 0.01). The $D_{\text{min}}$ values are not independent: for example, a single gene-flow event between ancestral lineages can affect multiple contemporary species and thus more trios than would a more recent gene-flow event. However, tree violations are numerous and pervasive throughout the dataset, within all the major groups and also between groups (Fig. 2a), revealing reticulate evolution at multiple levels. Therefore, phylogenetic trees alone cannot fully describe the evolutionary relationships of Lake Malawi cichlids.

**Phylogenetic framework.** Despite no tree giving a complete and accurate picture of the relationships between species, standard phylogenetic approaches are useful to provide a framework for discussion. To obtain an initial picture we divided the genome into 2,543 non-overlapping windows, each comprising 8,000 SNPs (average size 274 kb) and constructed a maximum likelihood phylogeny separately for the full sequences within each window, obtaining trees with 2,542 different topologies. We also calculated the maximum clade credibility (MCC) summary tree^31 and a maximum likelihood phylogeny based on the full mtDNA genome (Fig. 2b and Supplementary Fig. 6).

We next applied a range of further phylogenomic methods which are known to be robust to incomplete lineage sorting. These included three multispecies coalescent methods^34,35: the Bayesian SNAP^46 (with a subset of 48,922 unlinked SNPs in 12 individuals representing the eco-morphological groups), the algebraic method SVDquartets^27,28, which allows for site-specific rate variation and is robust to gene-flow between sister taxa^29, and the summary method ASTRAL^30,31, using the 2,543 local maximum likelihood trees that were described above as input. We also built a whole-genome neighbour-joining tree using the Dasarath et al. algorithm, which has been shown to be a statistically consistent and accurate species tree estimator under ILS^32,43. The above methods have also been applied to datasets where the individuals that are genetically intermediate between eco-morphological groups (C. trimaculatus, A. staughtrani and A. stевичи) have been removed, thus probably reducing the extent of violation of the multispecies coalescent model.

Despite extensive variation among the 2,543 individual maximum likelihood trees (at least in part attributable to ILS), and, to a lesser extent, variation between the different genome-wide phylogenetic methods, there is some general consensus (Fig. 2c and Supplementary Figs. 6–10). Except for the three previously identified intermediate species, individuals from within each of the previously identified eco-morphological groups cluster together in all the whole-genome phylogenies, forming well supported reciprocally monophyletic groups. The pelagic Diplotaxodon and Rhamphochromis together form a sister group to the rest of the radiation, except in the all-sample MCC and SVDquartets phylogenies. Perhaps surprisingly, all the methods place the generalist A. calliptera as the sister taxon to the specialized rocky-shore mbuna group in a position that is nested within the Lake Malawi radiation. On a finer scale, many similarities between the resulting phylogenies reflect features of previous taxonomic assignment, but some currently recognized genera are always polyphyletic, including Placidochromis, Lethrinops and Mylochromis.

The mtDNA phylogeny is an outlier, substantially different from all the whole-genome phylogenies and also from the majority of the local maximum likelihood trees (Fig. 2b,c and Supplementary Figs. 6 and 11). Discordances between mtDNA and nuclear phylogenies in Lake Malawi have been reported previously and interpreted as...
Fig. 2 | Excess allele sharing and patterns of species relatedness. a. Derived allele sharing reveals non-tree-like relationships among trios of species. The bars show the proportion of significantly elevated $D_{max}$ scores (see main text). Shading corresponds to FWER q values of (from light to dark) 10$^{-2}$, 10$^{-1}$, 10$^{-6}$ and 10$^{-14}$. The scatterplots show the $D_{max}$ scores that were significant with family-wise error rate (FWER) < 0.01. Results are shown separately for comparisons where all three species in the trio are from the same group, and for cases where the species come from two or three different groups. 

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b. A set of 2,543 maximum likelihood phylogenetic trees for non-overlapping regions along the genome. Branch lengths were scaled for visualization so that the total height of each tree is the same. The local trees were built with 71 species and then subsampled for display to 12 individuals representing the eco-morphological groups. 

The maximum clade credibility tree shown here was built from the subsampled local trees. A maximum likelihood mitochondrial phylogeny is shown for the same. The local trees were built with 71 species and then subsampled for display to 12 individuals representing the eco-morphological groups.

The scatterplots show the $D_{max}$ scores that were significant with family-wise error rate (FWER) < 0.01. Results are shown separately for comparisons where all three species in the trio are from the same group, and for cases where the species come from two or three different groups. Supplementary figures associated with the phylogenies are indicated for each tree.

A. stuartgranti and utaka within-group comparisons are not shown owing to the low number of data points. The maximum clade credibility tree shown here was built from the subsampled local trees. A maximum likelihood mitochondrial phylogeny is shown for the same. The local trees were built with 71 species and then subsampled for display to 12 individuals representing the eco-morphological groups.

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a signature of past hybridization events$^{16,20}$. However, as we discuss below, some of these previously suggested hybridization events are not reflected in the whole-genome data. Indeed, large discrepancies between mitochondrial and nuclear phylogenies have been shown in many other systems, reflecting both that mtDNA as a single locus is not expected to reflect the consensus under ILS, and high incidence of mitochondrial selection$^{44–46}$. This underlines the importance of evaluating species relationships in the Lake Malawi radiation from a genome-wide perspective.

Specific signals of introgression. We applied a variety of methods to identify the species and groups whose relationships violate the framework trees described in the previous section. First, we contrasted the pairwise genetic distances used to produce the

* trees that exclude samples with intermediate group assignment: C. trimaculatus, A. stenoni and A. stuartgranti

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neighbour-joining tree against the distances between samples along the tree branches, calculating the residuals (Supplementary Fig. 12). If the tree captured all the genetic relationships in our sample perfectly, the residuals would all be zero. However, as expected in light of the $D_{\text{man}}$ analysis above, we found numerous differences, affecting both groups of species and individual species, with some standout cases. Among the strongest signals on individual species, in addition to the previously discussed $C. \text{trimaculatus}$, we can see that (1) $\text{Placidochromis cf. longimanus}$ is genetically closer to the deep benthic clade and to a subset of the shallow benthic (mainly $\text{Lethrinops}$) species than the tree suggests; and (2) our sample of $\text{Otopharynx tetrastigma}$ (from Lake Ilamba) is much closer to $\text{A. calliptera}$ (especially to the sample from Lake Kingiri, only 3.2 km away) than is expected from the tree.

Second, the sharing of long haplotypes between otherwise distantly related species is an indication of recent admixture or introgression. To investigate this type of gene flow signature, we used the chromopainter software package and calculated the 'co-ancestry matrix' of all species—a summary of nearest-neighbour (therefore recent) haplotype relationships. The Lake Ilamba $O. \text{tetrastigma}$ and Lake Kingiri $\text{A. calliptera}$ also stand out in this analysis, showing a strong signature of recent gene flow between individual species from distinct eco-morphological groups (Supplementary Fig. 13). The other tree-violation signatures described above are also visible on the haplotype sharing level but are less pronounced, consistent with being older events involving the common ancestors of multiple present-day species. However, the chromopainter results indicate additional recent introgression events (for example, the utaka $C. \text{virginalis}$ with $\text{Diplotaxodon}$; more highlighted in Supplementary Fig. 13). Furthermore, the clustering based on recent co-ancestry is different from all phylogenetic trees: in particular a number of shallow benthics, including $C. \text{longimanus}$, cluster next to the deep benthics.

Third, we used the $f_1$ admixture ratio (f; closely related to Patterson’s $D$), computing $f(A,B,C,O)$ for all groups of species that fit the relationships $((A, B), C)$ in the ASTRAL* tree (Supplementary Fig. 7), with the outpost group fixed as $N. \text{brichardi}$. When elevated owing to introgression, the $f_1$ statistic is expected to be linear in relation to the proportion of introgressed material. The ASTRAL* tree has the lowest mean topological distance to all the other trees, and excludes the three species with intermediate group assignment, a choice made here because we were interested in identifying additional signals beyond the admixed status of $\text{A. stuartsgranti}$, $\text{A. steveni}$ and $C. \text{trimaculatus}$. Out of the 164,320 computed $f_1$ statistics, 97,889 were significant at FWER < 0.001.

As in the case of $D_{\text{man}}$, a single gene-flow event can lead to multiple significant $f_1$ statistics. Noting that the values for different combinations of $((A, B), C)$ groups are not independent as soon as they share branches on the tree, we sought to obtain branch-specific estimates of excess allele sharing that would be less correlated. Building on the logic employed to understand correlated gene flow signals in ref. , we developed the $f$-branch metric or $f_4(C)$: a summary of $f$ scores that, on a given tree, captures excess allele sharing between a species $C$ and a branch $b$ compared to the sister branch of $b$ (Methods). Therefore, an $f_4(C)$ score is specific to the branch $b$ (on the $y$-axis in Fig. 3), but a single introgression event can still lead to significant $f_4(C)$ values across multiple related $C$ values. There were 11,158 $f_4(C)$ scores of which 1,421 were significantly elevated at FWER < 0.001 (Supplementary Fig. 14), and 238 scores were larger than 3% (the value inferred for human–Neanderthal introgression in ref. )). The majority of nodes in the tree are affected: 92 of the 158 branches in the phylogeny show significant excess allele sharing with at least one other species $C$ (Fig. 3).

Overall, the highest $f_4(C)$ (14.2%) is between the ancestor of the two sampled $\text{Ctenopharynx}$ species from the shallow benthic group and the utaka $\text{Copadichromis virginalis}$ (Fig. 3). Notably, $\text{Ctenopharynx}$ species, particularly $\text{C. intermedius}$ and $\text{C. pictus}$, have very large numbers of long slender gill rakers, a feature shared with $\text{Copadichromis}$ species, and believed to be related to a diet of small invertebrates. Several other benthic lineages also share excess alleles with $\text{C. virginalis}$, however these signals are less pronounced. Next, the significantly elevated $f_4(C)$ scores between the shallow and the deep benthic lineages suggest that genetic exchanges between these two groups go beyond the clearly admixed shallow-living $\text{Aulonacara}$ (not included in this analysis). The $f$-branch signals between $O. \text{tetrastigma}$ and $\text{A. calliptera}$ Kingiri are observed in both directions—$\text{A. calliptera}$ Kingiri with shallow benthics (and most strongly $O. \text{tetrastigma}$) and $O. \text{tetrastigma}$ with $\text{A. calliptera}$ (most strongly $\text{A. calliptera}$ Kingiri), suggesting bi-directional introgression.

At the level of the major eco-morphological groups, the strongest signal indicates that the ancestral lineage of benthics and utaka shares excess derived alleles with $\text{Diplotaxodon}$ and, to a lesser degree, $\text{Rhamphochromis}$, as previously suggested by the PCA plot (Fig. 1c). Furthermore, there is evidence for additional ancestry from the pelagic groups in utaka, which could be explained either by an additional, more recent, gene-flow event or by differential fixation of introgressed material, possibly due to selection. Reciprocally, $\text{Diplotaxodon}$ shares excess derived alleles (relative to $\text{Rhamphochromis}$) with utaka and deep benthics, as does $\text{Rhamphochromis}$ with mbuna and $\text{A. calliptera}$. Furthermore, mbuna show excess allele sharing (relative to $\text{A. calliptera}$) with $\text{Diplotaxodon}$ and $\text{Rhamphochromis}$ (Fig. 3). On the other hand, while ref. suggested gene flow between the deep benthic and mbuna groups on the basis of a discrepancy between mtDNA and nuclear phylogenies, our genome-wide analysis did not find any signal of substantial genetic exchange between these groups.

The $f$-statistic tests are robust to the occurrence of incomplete lineage sorting, in the sense that ILS alone cannot generate a significant test result. We note, however, that pronounced population structure within ancestral species, coupled with rapid succession of speciation events, can also substantially violate the assumptions of a strictly bifurcating species tree and lead to significantly elevated $f$ scores. This needs to be taken into account when interpreting non-tree-like relationships, for example among major groups early in the radiation. However, in cases of excess allele sharing between ‘distant’ lineages that are separated by multiple speciation events, ancestral population structure would have needed to segregate through these speciation events without affecting sister lineages, a scenario that is not credible in general. Therefore, we suggest that there is strong evidence for multiple cross-species gene flow events. Additionally, simulations suggest that, compared with treemix, $f_4(C)$ is robust to misspecification of the initial tree (Supplementary Note).

Overall, the neighbour-joining tree residuals, the haplotype sharing patterns and the many elevated $f_4(C)$ scores paint a consistent picture. They confirm the extensive violations of the bifurcating species tree model initially revealed by the $D_{\text{man}}$ analysis, and suggest many independent gene-flow events at different times during the evolutionary history of the adaptive radiation.

**Origins of the radiation.** The generalist $\text{Astatotilapia calliptera}$ has been referred to as the ‘prototype’ for the endemic Lake Malawi cichlids and discussions concerning the origin of the radiation often centre on ascertaining its relationship to the Malawi species. Previous phylogenetic analyses, using mtDNA and small numbers of nuclear markers, showed inconsistencies in this respect. In contrast, our whole-genome data indicated a clear and consistent position of the Lake Malawi catchment $\text{A. calliptera}$ as a sister group to the mbuna, in agreement with the nuclear DNA phylogeny in a previous study. While it is not certain whether the 320 remaining mbuna species form a monophyletic group with the eight species we used here, the eight species represent the majority of the genera of...
Fig. 3 | Identifying tree violating branches and possible gene-flow events. The branch-specific statistic $f_0(C)$ identifies excess sharing of derived alleles between the branch of the tree on the y axis and the species $C$ on the x axis (see Supplementary Note). The ASTRAL* tree was used as a basis for the branch statistic and grey data points in the matrix correspond to tests that are not consistent with the phylogeny. Colours correspond to eco-morphological groups as in Fig. 1. Asterisks denote block jackknifing significance at $|Z| > 3.17$ (Holm–Bonferroni FWER < 0.001).

mbuna and therefore are likely to be representative of much of the genetic diversity within the group.

To explore the origins of the Lake Malawi radiation in greater detail, we obtained 24 additional Astatotilapia whole-genome sequences from outside Lake Malawi: five *A. calliptera* from Indian Ocean catchments, thus covering most of its geographical distribution, and 19 individuals from seven other Astatotilapia species (Supplementary Table 2). We generated new variant calls
Fig. 4 | Origins of the radiation and the role of A. calliptera. a, A neighbour-joining phylogeny showing the Lake Malawi radiation in the context of other East African Astatotilapia taxa. b, A Lake Malawi neighbour-joining phylogeny with expanded view of A. calliptera, with all other groups collapsed. c, Approximate A. calliptera sampling locations shown on a map of the broader Lake Malawi region. Black lines correspond to present-day level 3 catchment boundaries from the US Geological Survey’s HYDRO1K dataset (https://lta.cr.usgs.gov/HYDRO1K). d, Strong \( f_s \) admixture ratio signal showing that Malawi catchment A. calliptera are closer to mbuna than their Indian Ocean catchment counterparts. e, PCA of body shape variation of Lake Malawi endemics, A. calliptera and other Astatotilapia taxa, obtained from geometric morphometric analysis. f, A phylogeny with the same topology as in b but displayed with a straight line between the ancestor and A. calliptera. For each branch off this lineage, we show mean sequence divergence (\( d_{xy} \)) minus mean heterozygosity, and translation of this value into a mean divergence time estimate with 95% CI reflecting the statistical uncertainty in mutation rate. Dashed lines with arrows indicate likely instances of gene flow between major groups; their true timings are uncertain.

(Supplementary Methods) and first constructed a neighbour-joining tree, finding that all the A. calliptera (including Indian Ocean catchments) cluster as a single group nested at the same place within the radiation, whereas the other Astatotilapia species branched off well before the lake radiation (Fig. 4a–c). All A. calliptera individuals cluster by geography (Fig. 4b,c), except for the specimen from the crater lake Lake Kingiri, whose position in the tree is likely to be a result of admixture with O. tetrastigma. Indeed, a neighbour-joining tree built only with A. calliptera samples (Supplementary Fig. 17) places the Kingiri individual according to geography with the specimens from the nearby crater lake Lake Massoko and the Mbaka River.

Applying the same logic as above, we tested whether the position of the A. calliptera group in the neighbour-joining tree changes when the tree is built without mbuna (as would be expected if A. calliptera were affected by hybridization with mbuna). We found that the position of A. calliptera is unaffected (Supplementary Fig. 18), suggesting that the nested position is not due to later hybridization. The \( f \) statistics in Fig. 3 further support this, because the signals involving the whole mbuna or A. calliptera groups are modest and do not suggest erroneous placement of these groups in all phylogenetic analyses. Furthermore, the nested position of A. calliptera is also supported by the vast majority of the genome. Searching for the basal branch in a set of 2,638 local maximum likelihood phylogenies, we found results that agree with the whole-genome ASTRAL, SNAPP and neighbour-joining trees: the most common basal branches are the pelagic groups Rhamphochromis and Diplotoxodon (in 42.12% of the genomic windows). In comparison, A. calliptera (including Indian Ocean catchment samples) were found to be basal only in 5.99% of the windows (Supplementary Fig. 19).

Joyce et al.\(^{20}\) reported that the mtDNA haplogroup of A. calliptera from the Indian Ocean catchment clustered with mbuna (as we confirm in Supplementary Fig. 15) and suggested that there had been repeated colonization of Lake Malawi by two independent Astatotilapia lineages with different mitochondrial haplogroups: the first founding the entire species flock, and the second, with the Indian Ocean catchment mtDNA haplogroup, introgressing into the Malawi radiation and contributing strongly to the mbuna. This hypothesis predicts that, compared with the Malawi catchment A. calliptera, the Indian Ocean catchment A. calliptera should be closer to mbuna. However, across the nuclear genome we found a strong signal in the opposite direction, with 30% excess allele sharing between Malawi catchment A. calliptera and mbuna (Fig. 4d). Therefore, the Joyce et al.\(^{20}\) hypothesis that the mbuna, the most species-rich group within the radiation, may be a hybrid lineage formed from independent invasions is not supported by genome-wide data.

It has been repeatedly suggested that A. calliptera may be the direct descendant of the riverine-generalist lineage that seeded the Lake Malawi radiation\(^{12,50,53,54}\). Our interpretation of this argument...
is that the ancestor was probably a riverine generalist that was ecologically and phenotypically similar to *A. calliptera* and other *Astatotilapia*. This hypothesis is lent further support by geometric morphometric analysis. Using 17 homologous body shape landmarks we established that, despite the relatively large genetic divergence, *A. calliptera* is nested within the morphospace of the other more distantly related but ecologically similar *Astatotilapia* species (Fig. 4a,c), and these together have a central position within the morphological space of the Lake Malawi radiation (Fig. 4e and Supplementary Fig. 16).

To reconcile the nested phylogenetic position of *A. calliptera* with its generalist ‘prototype’ phenotype, we propose a model where the Lake Malawi species flock consists of three separate radiations splitting off from the lineage leading to *A. calliptera*. The relationships between the major groups supported by the ASTRAL, SNAPP and neighbour-joining methods suggest that the pelagic radiation was seeded first, then the benthic + utaka, and finally the rock-dwelling mbuna, all in a relatively quick succession, followed by subsequent gene flow as described above (Fig. 4f). The pelagic versus utaka + benthic branching order is swapped in the SVDquartets tree in Supplementary Fig. 9). Applying our per-generation mutation rate to observed genomic divergences we obtained mean divergence time estimates between these lineages between 460 thousand years ago (ka) (95% CI 350–990 ka) and 390 ka (95% CI 300–860 ka) (Fig. 4f), assuming three years per generation as in ref. 15. The point estimates all fall within the second-most recent prolonged deep-lake phase inferred from the Lake Malawi paleoecological record16, while the upper ends of the confidence intervals cover the third deep-lake phase at about 800 ka. Considering that our split time estimates from sequence divergence are likely to be reduced by subsequent gene-flow, leading to underestimates, the data are consistent with a previous report based on fossil time calibration which put the origin of the Lake Malawi radiation at 700–800 ka17.

The fact that the common ancestor of all the *A. calliptera* appears to be younger than the Malawi radiation suggests that the Lake Malawi *A. calliptera* population has been a reservoir that has repopulated the river systems and more transient lakes following dry–wet transitions in the East African hydroclimate18–20. Our results do not fully resolve whether the lineage leading from the common ancestor to *A. calliptera* retained its riverine generalist phenotype throughout or whether a lacustrine species evolved at some point (for example, the common ancestor of *A. calliptera* and mbuna) and later de-specialized again to recolonize the rivers. However, while it is a possibility, we suggest that it is unlikely that the many strong phenotypic affinities of *A. calliptera* to the basal *Astatotilapia* (Fig. 4c; refs 13,20) would be reinvented from a lacustrine species.

**Signatures and consequences of selection on coding sequences.** To gain insight into the functional basis of diversification and adaptation in Lake Malawi cichlids, we next turned our attention to protein-coding genes. We compared the mean between-species levels of non-synonymous variation $\delta_{N-S}$ to synonymous variation $\delta_{S}$ in 20,664 genes and calculated the difference between these two values ($\Delta_{N-S} = \delta_{N-S} - \delta_{S}$). Overall, coding sequence exhibits signatures of purifying selection: the average between-species $\delta_{N-S}$ was 54% lower than in a random matching set of non-coding regions. Interestingly, the relative enrichment in this category is both substantial and highly significant (Fig. 5b). On the other hand, the increase in proportion of candidate genes in the $N = 1$ category (multiple copies in the *M. zebra* genome but only one copy in zebrafish) is much smaller and is not significant ($\chi^2$ test $P = 0.18$), suggesting that selection is occurring more often within ancient multi-copy gene families, rather than on genes with cichlid-specific duplications.

We used GO annotation of zebrafish homologues to test whether candidate genes are enriched for particular functional categories (Methods). We found significant enrichment for 30 GO terms (range: 1.6 × 10⁻⁸ < $P < 0.01$, weigh algorithm21, Supplementary Table 3): 10 in the ‘molecular function’, 4 in the ‘cellular component’ and 16 in the ‘biological process’ category. Combining all the results in a network (connecting terms that share many genes) revealed clear clusters of enriched terms related to (1) haemoglobin function and oxygen transport; (2) phototransduction and visual perception; and (3) the immune system, especially inflammatory response and cytokine activity (Fig. 5c). That evolution of genes in these functional categories has contributed to cichlid radiations has been suggested previously (see below); it is nevertheless interesting that these categories stand out in a genome-wide analysis.

**Shared mechanisms of depth adaptation.** To gain insight into the distribution of adaptive alleles across the radiation, we built maximum likelihood trees from amino acid sequences of candidate genes, thus summarizing potentially complex haplotype genealogy networks. Focusing on the significantly enriched GO categories, many haplotype trees have features that are unusual in the broader dataset: the haplotypes from the deep benthic group and the deep-water pelagic *Diplotaxodon* tend to group together (despite these two groups being distant in whole-genome phylogenies and monophyletic in only two out of 2,638 local maximum likelihood trees) and also tend to be disproportionately diverse when compared with the rest of the radiation. We quantified both excess similarity and diversity, and found that both measures are elevated for candidate genes in the ‘visual perception’ category (Fig. 6a; Mann–Whitney U-tests: $P = 0.007$ for similarity, $P = 0.08$ for shared diversity, and $P = 0.003$ when the scores are added) and also for the ‘haemoglobin complex’ category ($P$ values not significant owing to the small number of genes).

Sharply decreasing levels of dissolved oxygen and low light intensities with narrow short-wavelength spectra are the hallmarks of the habitats below about 50 m to which the deep benthic and *Diplotaxodon* groups have both adapted, either convergently or in...
Shared signatures of selection in genes involved in vision and in oxygen transport therefore point to shared molecular mechanisms underlying this ecological parallelism. Further evidence of shared mechanisms of adaptation is that, for genes annotated with ‘photoreceptor activity’ and ‘haemoglobin complex’ GO terms, the $\Delta_{\text{NS},5}$ selection score is strongly correlated with the local levels of excess allele sharing between the two depth-adapted groups measured by the $f_{\text{NS}}$ statistic, a conservative version of the $f$ statistic more suited to analysing small genomic intervals (Fig. 6b; $\rho_s = 0.63$ and 0.81, $P = 0.001$ and $P = 0.051$, respectively).

Vision genes with high similarity and diversity scores for the deep benthic and Diplotaxodon groups include three opsins: the green-sensitive RH2Aβ and RH2B, and rhodopsin (Fig. 6a and Supplementary Fig. 20). The specific residues that distinguish the deep benthic and Lake Malawi reference (M. zebra) are expressed exclusively in double-cone photoreceptors, RH2B, and rhodopsin (Fig. 6c) (Supplementary Note).

The functions of these genes, together with the fact that RH2Aβ and RH2B are expressed exclusively in double-cone photoreceptors, suggest a prominent role of cone-cell vision in depth adaptation. The wavelength of maximum absorbance in cone cells expressing RH2Aβ (498 nm) corresponds to the part of the visible-light spectrum that best transmits into deep water in Lake Malawi.

There have been many studies of selection on opsin genes in fish, including selection associated with depth preference, but having whole-genome coverage allows us to investigate other components of primary visual perception in an unbiased fashion. We found shared patterns of selection between deep benthics and Diplotaxodon in six other vision-associated candidate genes (Fig. 6a). The functions of these genes, together with the facts that RH2Aβ and RH2B are expressed exclusively in double-cone photoreceptors, suggest a prominent role of cone-cell vision in depth adaptation. The wavelength of maximum absorbance in cone cells expressing a mixture of RH2Aβ with RH2B (498 nm) corresponds to the part of the visible-light spectrum that best transmits into deep water in Lake Malawi.
Fig. 6 | Shared selection between the deep-water-adapted groups *Diplotaxodon* and deep benthic. a, The scatterplot shows the distribution of genes with high ΔN-S scores (candidates for positive selection) along axes reflecting shared selection signatures. Only genes with zebrafish homologues are shown. Amino acid haplotype trees, shown for genes as indicated by the red symbols and numbers, indicate that *Diplotaxodon* and deep benthic species are often divergent from other taxa, but similar to each other. Outgroups include *Oreochromis niloticus*, *Neolamprologus brichardi*, *Astatotilapia burtoni*, and *Pundamilia nyererei*. b, Selection scores plotted against \( f_{\text{sig}} \) (mbuna, deep benthic; *Diplotaxodon*, *N. brichardi*), a measure of local excess allele sharing between deep benthic and *Diplotaxodon*\(^\text{\textregistered}\). c, A schematic drawing of a double cone photoreceptor expressing the green-sensitive opsins and illustrating the functions of other genes with signatures of selection. d, \( f_{\text{sig}} \) calculated in sliding windows of 100 SNPs around the green opsin cluster, revealing that excess allele sharing between deep benthic and *Diplotaxodon* extends far beyond the coding sequences.

Figure 6c illustrates interactions of the vision genes with shared selection patterns in the cichlid double-cone photoreceptor. The homeobox protein six7 governs the expression of RH2 opsins and is essential for the development of green cones in zebrafish\(^\text{\textregistered}\) (specific mutations are highlighted in Supplementary Fig. 20). The kinase GRK7 and the retinal cone arrestin-C have complementary roles in photoreceptor activity: arrestin produces the final shutoff of the photoreceptor activity \( \text{GO} \). The shared patterns of depth selection may be particular to the *Diplotaxodon* and the deep benthic group. e, A schematic drawing of a double cone photoreceptor expressing the green-sensitive opsins and illustrating the functions of other genes with signatures of shared selection. f, \( f_{\text{sig}} \) calculated in sliding windows of 100 SNPs around the green opsin cluster, revealing that excess allele sharing between deep benthic and *Diplotaxodon* extends far beyond the coding sequences.

Also noted was the similarity of the haemoglobin subunit beta (\( \text{HB}\beta \)) haplotypes between *Diplotaxodon* and deep benthic species. We confirmed signatures of selection in the two annotated LA cluster haemoglobins. In addition, we found that four haemoglobin subunits (\( \text{HB}\beta1, \text{HB}\beta2, \text{HBA}2 \) and \( \text{HBA3} \)) from the MN cluster are also among the genes with high selection scores (Supplementary Fig. 22). The shared patterns of depth selection may be particular to the \( \beta \)-globin genes (Supplementary Fig. 22), although this hypothesis remains tentative, because the repetitive nature of the MN cluster precludes us from confidently examining all haemoglobin genes.

A key question concerns the mechanism leading to the similarity of haplotypes in *Diplotaxodon* and deep benthic. Possibilities include parallel selection on variation segregating in both groups owing to common ancestry, selection on the gene flow that we described in a previous section, or independent selection on new mutations. Considering the haplotype trees and local patterns of excess allele sharing (using \( f_{\text{sig}} \) statistics\(\text{\textregistered}\)), there is evidence for each of these processes acting on different genes. The haplotype trees for rhodopsin and \( \text{HB}\beta \) have outgroup taxa (and also *A. calilpera*) appearing at multiple locations on their haplotype networks (Fig. 6a), suggesting that the haplotype diversity of these genes
may reflect ancestral variation. In contrast, trees for the green cone genes show the Malawi radiation all being derived with respect to outgroups and we found substantially elevated $f_{st}$ scores extending for around 40 kb around the RH2 cluster (Fig. 6d), consistent with adaptive introgression in a pattern reminiscent of mimicry loci in Heliconius butterflies. Finally, the peaks in $f_{st}$ around peripherin-2 and one of the arrestin-C genes are narrow, ending at the gene boundaries, and $f_{st}$ scores are elevated only for non-synonymous variants; synonymous variants do not show excess allele sharing (Supplementary Fig. 23). Owing to the close proximity of non-synonymous and synonymous sites within the same gene, this suggests that for these two genes there may have been independent selection on the same de novo mutations.

**Discussion**

Variation in genome sequences forms the substrate for evolution. Here we described genome variation at the full sequence level across the Lake Malawi haplochromine cichlid radiation. We focused on ecomorphological diversity, representing more than half the genera from each major group, rather than obtaining deep coverage of species within any particular group. Therefore, we have more samples from the morphologically highly diverse benthic lineages than, for example, from the mbuna where there are relatively fewer genera and many species are largely recognized by colour differences.

The observation that cichlids within an African Great Lake radiation are genetically very similar is not new, but we now quantify the relationship of this to within-species variation, and the consequences for variation in local phylogeny across the genome. The fact that between-species divergence is generally only slightly higher than within species diversity, is probably the result of the young age of the radiation, the relatively low mutation rate and of gene flow between taxa. Within-species diversity itself is relatively low for vertebrates, at around 0.1%, suggesting that low genome-wide nucleotide diversity levels do not necessarily limit rapid adaptation and speciation, results that are in contrast to a recent report that found that high diversity levels may have been important for rapid adaptation in Atlantic killifish. One possibility is that in cichlids repeated selection has maintained diversity in adaptive alleles for a range of traits that support ecological diversification, as we have concluded for rhodopsin and HBβ and as appears to be the case for some adaptive variants in sticklebacks.

We provide evidence that gene flow during the radiation, although not ubiquitous, has certainly been extensive. Overall, the numerous violations of the bifurcating species tree model suggest that full resolution of interspecies relationships in this system will require network approaches (see for example section 6.2 of ref. 18) and population genomic analyses within the structured coalescent framework with gene flow. The majority of the signals affect groups of species, suggesting events involving their common ancestors, or are between closely related species within the major ecological groups. The only strong and clear example of recent gene flow between individual distantly related species is not within Lake Malawi itself, but between Otopharynx tetrasigma from crater Lake Ilamba and local A. calliptera. Lake Ilamba is very turbid and the scenario is reminiscent of cichlid admixture in low-visibility conditions in Lake Victoria. It is possible that some of the earlier signals of gene flow between lineages we observed in Lake Malawi may have happened during periods of low lake level when the water is known to have been more turbid.

Our model of the early stages of radiation in Lake Malawi (Fig. 4f) is broadly consistent with the model of initial separation by major habitat divergence, although we propose a refinement in which there were three relatively closely spaced separations from a generalist Astatotilapia type lineage, initially of pelagic genera Rhamphochromis and Diplotaxodon, then of shallow- and deep-water benthics and utaka (this includes Kocher's sand dwellers19,29), and finally of mbuna. Thus, we suggest that Lake Malawi contains three separate haplochromine cichlid radiations stemming from the generality of lineage, interconnected by subsequent gene flow.

The finding that cichlid-specific gene duplicates do not tend to diverge particularly strongly in coding sequences (Fig. 5b) suggests that other mechanisms of diversification following gene duplications may be more important. Divergence via changes in expression patterns has previously been illustrated and discussed, and future studies addressing structural variation between cichlid genomes will assess the contribution of differential retention of duplicated genes.

The evidence concerning shared adaptation of the visual and oxygen-transport systems to deep-water environments between deep benthics and Diplotaxodon suggests different evolutionary mechanisms acting on different genes, even within the same cellular system. It will be interesting to see whether the same genes or even specific mutations underlie depth adaptation in Lake Tanganyika, which harbours specialist deep-water species in least two different tribes and has a similar light attenuation profile but a steeper oxygen gradient than Lake Malawi.

Over the last few decades, East African cichlids have emerged as a model for studying rapid vertebrate evolution. Taking advantage of recently assembled reference genomes, our data and results provide insight into patterns of sequence sharing and adaptation across the Lake Malawi radiation, and into mechanisms of rapid phenotypic diversification. The datasets are publicly available (see ‘Data availability’) and will underpin further studies on specific taxa and molecular systems. For example, we envisage that our results, clarifying the relationships between all the main lineages and many individual species, will facilitate speciation studies, which require investigation of taxon pairs at varying stages on the speciation continuum and studies on the role of adaptive gene flow in speciation.

**Methods**

**Samples.** Ethanol-preserved fin clips were collected by M. J. Genner and G. F. Turner between 2004 and 2014 from Tanzania and Malawi, in collaboration with the Tanzania Fisheries Research Institute (the MolEcoFish Project) and the Fisheries Research Unit of the Government of Malawi (various collaborative projects). Samples were collected and exported with the permission of the Tanzania Commission for Science and Technology, the Tanzania Fisheries Research Institute, the Fisheries Research Unit of the Government of Malawi.

From sequencing to a variant callset. The analyses presented above are based on SNPs obtained from Illumina short (100–125 bp) reads, aligned to the M. zebra reference assembly version 1.1 with bwa-mem, followed by GATK haplotype caller and samtools/bcftools variant calling restricted to 653-Mb of ‘accessible genome’ where variants can be determined confidently with short reads, filtering, genotype refinement, imputation and phasing in BEAGLE and further haplotype phasing with shapet v2, including the use of phase-informative reads. For details please see Supplementary Methods.

**Linkage disequilibrium calculations.** The haplotype disequilibrium coefficient $r^2$ between pairs of SNPs was calculated along the phased scaffolds 0 to 201 (scaffolds are assembled fragments of the reference genome and scaffolds 0–201 are longer than 1 Mb), using vcftools v0.1.12b with the options --hap-r2 --ld-window-bp 50000. To reduce the computational burden, we used a random subsample of 10% of SNPs. We binned the $r^2$ values according to the distance between SNPs into 1-kb or 100-bp windows and plotted the average values in each bin.

To estimate background linkage disequilibrium, we calculated haplotype $r^2$ between variants mapping to different linkage groups in the Oreochromis niloticus genome assembly. First, we used the chain files generated by the whole genome alignment pipeline (see Supplementary Methods) and the UCSC liftOver tool (http://hgdownload.soe.ucsc.edu/downloads.html#source_downloads) to translate the genomic coordinates of all SNPs to the O. niloticus coordinates. Then we calculated linkage disequilibrium between variants mapping to linkage groups LG1 and LG2.

**De novo mutation rate estimation.** In each trio we looked for mutations in the child that were not present in either of its parents. Because the results of this analysis are very sensitive to false positives and false negative rates, we used higher coverage sequencing (about 40x average) and applied more stringent genome masks than in the population genomic work. Increased coverage supports clean separation of sequencing errors and somatic mutations from true heterozygous
calls in the offspring, and improved ability to distinguish single copy versus multi-
copy sequence on a per-individual basis.

First we determined the accessible genome (that is the regions of the genome in
which the mutations can be confidently called (de novo mutations) for each trio by
excluding:
1. Genomic regions where mapped read depth in any member of a trio is ≤25x or
   >50x.
2. Bases where either of the parents has a mapped read that does not match the
   reference (the specific bases where any read has no reference alleles in the
   parents were masked).
3. Sequences where indels (base insertion or deletion) were called in any sample
   (we also excluded ≥3bp of sequence surrounding the indel).
4. Sites that were called as multiallelic among the nine samples in the overall
   trios dataset.
5. Known segregating variable sites—that is, sites with alternative alleles found
   in four and more copies in the overall Lake Malawi dataset.
6. Sites in the reference where less than 90% of overlapping 50-mers (sub-sequences of length 50) could be matched back uniquely and without
   difference. For this we used Heng Li’s SnPable tool (http://loh3.h3.users.
   sourceforge.net/snpsnape.shtml), dividing the reference genome into overlapping
   k-mers (sequences of length k; we used k = 50), and then aligning the extracted k-mers back to the genome (we used
   bwa aln -R 1000000 -o 3 -E 3).

After excluding sites in the categories above, we were left with an accessible genome of 516.6 Mb in the A. calliptera trio, 459 Mb in the A. stuartgranti trio and
404 Mb in the L. letrinus trio. Because any observed de novo mutation could have
occurred either on the chromosome inherited from the mother or on the
chromosome inherited from the father, the point estimate of the per-generation
per-base pair mutation rate is \( \mu = \frac{N_m}{2N_e} \times \frac{1}{2} \times \text{the size of the accessible genome} \).

Next we set out to search for de novo mutations: that is, heterozygous sites in
the offspring within the accessible genome. Under random sampling there is an
equal probability of seeing a read with either of the two alleles at a heterozygous
site. Therefore, \( \frac{N}{k} \), (the number of reads supporting the alternative allele) is
distributed as approximately Binomial(read depth, 0.5). We filtered out variants
with observed \( \frac{N}{k} \) values below the 2.5th or above the 97.5th percentiles of this
distribution, thus accepting a false-negative rate of 5%. We also filtered out sites
with observed \( \frac{N}{k} \) distributed as approximately Binomial(read depth, 0.5). We filtered out variants
because of uneven sequencing depth, we limited these analyses to one high-coverage
(15x) individual per species. Species with a high-coverage sample (\( R_{subcloris}, F_{rostratus} \) and \( L. \) trewavasae) were not included.

Outgroup sequences/alleles. Outgroup (Supplementary Table 5) sequences in
M. zebra genomic coordinates were obtained based on pairwise whole-genome
alignments (Supplementary Methods). Insertions in the outgroup were ignored
and deletions filled by ‘N’ characters.

Local phylogenetic trees and maximum clade credibility. To generate a multiple
alignment input in fasta format we used the getWGSep subprogram of evo. We set
the window size in terms of the numbers of variants rather than physical length
(8,000 variants; the \( \text{--split } \) option) aiming for the local regions to have similar
strengths of phylogenetic signal. Small windows at the ends of scaffolds were
discarded. We limited the sequence output to the accessible genome using the
--accessibleGenomeBED option. The N. brichardi outgroup sequence was added via
the --inB-Pn option.

Maximum likelihood phylogenies were inferred using RAxML version
7.7.89 under the GTRGAMMA model. The best tree for each region was
selected out of twenty alternative runs on distinct starting maximum parsimony
trees (the \( -N \) 20 option).

The MCC trees were calculated in TreeAnnotator version 2.4.2, a part of the
BEAST2 platform. The MCC tree is the tree (from among the trees in the set) that
maximizes the product of the frequencies of all its clades. For the non-overlapping
regions the MCC tree is a summary as a reference of the frequencies of each clade
in the whole set via the ‘common ancestor’ heights option.

Mitochondrial DNA phylogenies. The mtDNA sequence corresponds to
scaffolds 747 and 2,036 in the M. zebra reference. Variants from these scaffolds
were subjected to the same filtering as in the rest of the genome except for the
depth filter because the mapped read depth was much higher (approximately 300–400x per sample). Because of the greater sequence diversity in the mtDNA
data set, we found that more than 10% of variants were multiallelic. Therefore,
we separated SNPs from indels at multiallelic sites using bcftools norm with the
--multiallelic option, then removed indels and the merged multiallelic SNPs
back together with the --multiallelic + option. Sequences in the fasta format
were generated using the bcftools consensus command, and missing genotypes
in the VCF replaced by the ‘N’ character with the --mask option. The N. brichardi
outgroup sequence in M. zebra genomic coordinates was added to the fasta files.
A maximum likelihood tree was inferred using RAxML version 7.7.89 under
the GTRGAMMA model. The best tree was selected out of twenty alternative runs
on distinct starting maximum parsimony trees (using the \( -N \) 20 option) and two
hundred bootstrap replicates were obtained using RAxML rapid bootstrapping
algorithm satisfying the \(-Na\) auto frequency-based bootstrap stopping criterion.
Bipartition bootstrap support was drawn on the maximum likelihood tree using
RAxML -b option.

Genome-wide Fs calculations. In addition to performing PCA, the smartpca
program from the eigensoft v5.0.2 software package with default parameters.

Genome-wide Fs calculations. In addition to performing PCA, the smartpca
program from the eigensoft v5.0.2 software package also calculates genome-wide
Fs for fixed pairs of populations specified by the sixth column in the .ped
file. For the calculation, it uses the Hudson estimator, as defined previously
in their equation (10), and the \( \sqrt{\text{average} \times \text{of}} \) is used to calculate the sum of estimates of \( \overline{\text{Fs}} \)
across multiple variants, as they recommended. We used all SNPs (no minor allele
frequency filtering).

Allele sharing test for group assignment. We tested whether two individuals who
come from the same group always share more derived alleles with each other than
with any individuals from other groups. Technically, we implemented this using the
D statistic (ABRA-BABA tests) framework\(^{14,15} \), by calculating \( \text{D(A,G, O)} \) for
all permutations of individuals, where \( G_i \) and \( G_j \) come from the same
eco-morphological group and \( A \) from a different group. The outgroup \( O \) was always
N. brichardi from Lake Tanganyika. Note that this is an unusual use of the
D statistics and our aim here was not to look for gene flow but to test whether
allele sharing is greater within eco-morphological groups (\( G_i \) with \( G_j \)) compared
across groups (\( A \) with \( G_i \)), in which case \( \text{D(A,G, G, O)} > 0 \). All results were
statistically significant, which was assessed using block jackknife \(^\ast \) on windows of
60,000 SNPs.

The D\( _{\text{AB}} \) statistic. Here we calculated the D statistic for each trio of species (\( A,B,C \)) and
for all possible tree topologies (the outgroup again fixed as N. brichardi). Therefore,
D\( _{\text{AB}} = \min(\text{D}(A,B,C,O), \text{D}(A,C,B,O), \text{D}(C,B,A,O)) \). If this is significantly elevated,
then allele sharing within the trio of species is inconsistent with any simple tree
 topology. Note that this approach is conservative in the sense that the D\( _{\text{AB}} \) score
for each trio is considered in isolation and we ignore ‘higher-order’ inconsistencies
where different D\( _{\text{AB}} \) trio topologies are inconsistent with each other. Statistical significance
was assessed using block jackknife \(^\ast \) on windows of 60,000 SNPs and family wise error
rate (FWER) was calculated following the Holm–Bonferroni method.

Sample selection for demographic analyses. To prevent potential confounding
effects of uneven sequencing depth, we limited these analyses to one high-coverage
(15x) individual per species. Species without a high-coverage sample (\( R_{subcloris}, F_{rostratus} \) and \( L. \) trewavasae) were not included.
We measured the distances between all pairs of species in the reconstructed neighbour-joining tree (that is the lengths of branches) using the get_distance() method implemented in the ETREE toolkit for phylogenetic trees. Our first measure of ‘tree violation’ is the difference between these distances and the distances between samples in the original matrix that was used to build the neighbour-joining tree.

**Multiples species coalescent methods.** We applied three different methods that attempt to reconstruct the species tree under the multiples species coalescent model. For a brief discussion of these approaches see Supplementary Methods.

For SNAP46 we used a random subset of about 0.5% of genome-wide SNPs (48,922 SNPs) for 12 individuals representing the eco-morphological groups and the Lake Victoria outgroup *P. nyererei*, whose alleles were filled in based on the whole-genome alignment. The *P. nyererei* alleles were assigned as ‘ancestral’ (0) in the *Danio* input, the ‘forward’ or ‘backward’ mutation parameters u and v were calculated directly from the data by SNAPP (the ‘Calc mutation’ option). The default value 10 was used for the ‘Coalescent rate’ parameter and the value of the parameter was sampled (estimated in the Markov chain Monte Carlo (MCMC) chain). We used uninformative priors as we do not assume strong a priori knowledge about the parameters. The prior for ancestral population sizes was chosen to be a relatively broad gamma distribution with parameters a = 4 and β = 20. The tree height prior λ was set to the initial value of 100 but sampled in the MCMC chain with an uninformative uniform hyperprior on the interval [0, 50,000]. We ran three independent MCMC chains with the same starting parameters, each on 30 threads with a total runtime of over 10 central processing unit (CPU) years. The first one million steps from each MCMC chain was discarded as burn-in. In total, more than 30 million MCMC steps were sampled in the three runs. For the MCMC traces for each run, see Supplementary Fig. 24.

Next we used SVDquartets37,38 as implemented in PAUP* (v4.0a, build 1594). We prepared the data in the NEUS ‘.dta’ format, using evo with the getWGSseq --whole-genome --makeSVInput -r options. This command outputs for each individual the data base at each variable site, randomly sampling one of the two alleles at heterozygous sites, and ignoring sites that become monomorphic owing to this random sampling of alleles. The final dataset contained 17,853,187 SNPs. Then we ran SVDquartets in PAUP* setting outgroup to *N. brichardi* and then executing svdq eval-quartet—all specifying that all quartets should be evaluated (not just a random subset). In the final step, PAUP* version of the QEM algorithm103 is used to search for the overall tree that minimizes the number of quartets that are inconsistent with it.

Finally we used ASTRAI (v.5.6.1) with default parameters and the full set of 2,543 local trees generated by RAXML (see above) as input.

**Tree comparisons.** To summarize the degree of (dis)agreement between the topologies of trees produced by different phylogenetic methods (Fig. 2c), we calculated the normalized Robinson–Foulds distances between pairs of trees104 using the ReDist function from the phangorn package in R with the option normalize=TRUE.

**Chromopainter and fineSTRUCTURE.** Singleton SNPs were excluded using the bcftools v1.1 <2 minor option, before exporting the remaining variants in the PLINK format1. The chromopainter v0.4.0 software was then run for the 201 largest genomic scaffolds on shape-shuffled SNPs. Briefly, we created a uniform recombination map using the makeunimorphicreflce.pl script, then estimated the effective population size (N_e) for a subsample of 20 individuals using the chromopainter inbuilt expectation-maximization procedure22, averaged over the 20 N_e values using the provided neaverage.pl script. The chromopainter program was then run for each scaffold independently, with the -a 0 option to run all individuals against all others. Results for individual scaffolds were combined using the chromosome tool before running fineSTRUCTURE v0.6.5 with 1,000,000 burn-in iterations, and 200,000 sample iterations, recording a sample every 1,000 iterations (options -x 1000000 -y 200000 -z 1000). Finally, the sample relationship tree was built with fineSTRUCTURE using the -m T option and 20,000 iterations.

**The μ-branch statistic.** The f4-admixture ratio (f(4) statistic) was developed to estimate the proportion of introgressed material in an admixed population (see SOM18 in ref. 1 and f4 in ref. 1). However, when calculated for different subsets of samples within the same phylogeny, there are a very large number of highly correlated f values that are hard to interpret. To make the interpretation easier, we developed the ‘μ-branch’ metric or \( f_{μ}(C) = \mu \cdot \text{median} [\min_{j} [f(A, B; C, O)] \] , where \( B \) are samples descending from branch \( b \), and \( A \) are samples descending from the sister branch of \( b \). The outgroup \( O \) was always \( N. brichardi \). The \( f_{μ}(C) \) score provides for each branch \( b \) of a given phylogeny and each sample \( C \) a summary of excess allele sharing of branch \( b \) with sample \( C \) (Fig. 3, Supplementary Fig. 26). Each \( f_{μ}(C) \) score was also assigned an associated z-score to assess statistical significance \( Z_{C}(f_{μ}) = \text{median} [\min_{j} [Z(A, B; C, O)] \] . Additional information on the \( f \) and \( f_{μ}(C) \) statistics, including detailed reasoning behind the design of \( f_{μ}(C) \), are in Supplementary Methods.

**Geometric morphometric analyses.** A total of 168 photographs were used to compare the gross body morphology of *Astatotilapia calliptera* to that of endemic Lake Malawi species and other East African *Astatotilapia* lineages (Supplementary Table 7). Coordinates for 17 homologous landmarks (following ref. 17) were collected using tpsDig2 v2.26.46. After landmark digitization, analysis of shape variation was carried out in R (v3.3.2) using the package Geomorph v3.0.2.45. First a General Procrustes Analysis was applied to remove non-shape variation and shape data were corrected for allometric size effects by performing a regression of Procrustes coordinates (10,000 iterations). The resulting allometry-corrected residuals were used in PCA.

**Maps.** Present-day catchment boundary maps are based on ‘level 3’ detail of the Hydro1K dataset from the US Geological Survey. We downloaded the watershed boundary data from the United Nations Environment Programme website (http://edc.grid.unep.ch) and processed it using the QGIS geographic information system software (http://www.qgis.org/en/site/).

**Protein-coding gene annotations.** We used the BROADDMZ2 annotation generated by the cichlid genome project1 and removed overlapping transcripts using Jim Kent’s genePredSingleCover program. Genes whose annotated length in nucleotides was not divisible by three were discarded, as they typically had inaccuracies in annotation that would require manual curation (2,495 out of 23,698 genes). We also used the cichlid genome project1 assignment of homologues between the *M. zebra* genome reference and zebrasfish (Danio rerio).

**Coding sequence positive selection scan.** We used evo with the getCodingSeq -Hb -tostats option to obtain the coding sequences for each allele and each gene. The excess of non-synonymous variation (\( \delta_n-s \)) and the non-synonymous variation excess score (\( \delta_{N-s} \)) were calculated on a per-gene basis as follows. Let \( N_s \) be the number of possible non-synonymous transitions and \( N_t \) the number of possible non-synonymous transversion between two sequences; analogously \( S_s \) and \( S_t \) represent possible synonymous differences. We do not specify the ancestral allele, and therefore consider it equally likely that allele \( i \) mutated into allele \( j \) or that allele \( j \) mutated to allele \( i \). Then let \( N \) be the number of observed non-synonymous mutations and \( S \) the number of observed synonymous mutations. If there is more than one difference within a codon, all ‘mutation pathways’ (that is, the different orders in which mutations could have happened) have equal probabilities. When a particular allele contained a premature stop codon, the remainder of the sequence after the stop was excluded from the calculations.

Because the transition/transversion ratio in the Lake Malawi dataset was 1.73, and hence (because there are two possible transversions for each possible transition) the prior probability of each transition is 3.46 times that of each transversion, we account for the unequal probabilities of transitions and transversions in calculating the proportions of non-synonymous (\( p_n \)) and of synonymous differences (\( p_s \)) as follows:

\[
p_n = \frac{N}{S} = \frac{3.46 \times N_{ts} + N_{tv}}{S} \quad p_s = \frac{S}{3.46 \times N_{ts} + S_{tv}}
\]

The excess of non-synonymous variation (\( \delta_{n-s} \)) is the average of \( p_n - p_s \) over pairwise sequence comparisons. Only between-species sequence comparisons are considered for the Lake Malawi dataset. We normalized the \( \delta_{n-s} \) values in order to take into account the effect on the variance of this statistic introduced by differences in gene length and by sequence composition. To achieve this, we used the leave-one-out jackknife procedure across different pairwise comparisons for each gene, estimating the standard error. The non-synonymous variation excess score (\( \delta_{N-s} \)) is then:

\[
\delta_{N-s} = \frac{\delta_{n-s}}{\text{jackknife}_{se}(\delta_{n-s})}
\]

Note that because the sequences are related by a genealogy, there is a correlation structure between the pairwise comparisons. Therefore, the jackknife approach substantially underestimates the true standard error of \( \delta_{N-s} \), and is used here simply as a normalization factor.

The null model shown in Fig. 5a was derived by splitting all the coding sequence into its constituent codons, and then randomly sampling these codons with replacement to build new sequences that matched the actual coding genes in their numbers and the length distribution. Then we calculated the \( \delta_{N-s} \) scores, as we did for the actual genes and compared the two distributions. High positive values at the upper tail of the distribution are substantially over-represented in the actual data when compared to a null model.

We also calculated the above statistics for random non-coding regions, matching the gene sequences in length. We used the bedtools v2.26.44 ‘shuffle’ command to permute the locations of exons along the chromosomes. Of the total length of all the permuted sequences, 98.4% were within the ‘accessible genome’ and outside coding sequences (we required at least 95% in any of the
permitted locations). The specific command was bedtools shuffle -chrom -e exons.

**GO enrichment.** Zebrafish has the most extensive functional gene annotation of any fish species, providing a basis for GO enrichment analysis. GO enrichment for the genes that were candidates for being under positive selection (the top 5% of $\Delta_{\text{raw}}$ values) was calculated in R using the topGO v2.26.0 package\(^{41}\) from the Bioconductor project\(^{42}\). The GO hierarchical structure was obtained from the GO.db v3.4.0 annotation and linking zebrafish gene identifiers to GO terms was accomplished using the org.Dr.gencode v3.4.0 genome-wide, between 9,024 and 9,353 genes had a GO annotation that could be used by topGO, the exact number depending on the GO category being assessed. The nodeSize parameter was set to 5 to remove GO terms which have fewer than five annotated genes, as suggested in the topGO manual.

There is often an overlap between gene sets annotated with different GO terms, in part because the terms are related to each other in a hierarchical structure\(^{41}\). This is partly accounted for by our use in topGO of the weight algorithm that accounts for the GO graph structure by down-weighing genes in the GO terms that are neighbours of the locally most significant terms in the GO graph\(^{41}\). All the P values we report are from the weight algorithm, which the authors suggest should be reported without multiple testing correction\(^{11}\).

Some interdependency between significant GO terms remains after using the weight algorithm. Therefore, we used the Enrichment Map\(^{5}\) app for Cytoscape (http://www.cytoscape.org) to organize all the significantly enriched terms into networks where terms are connected if they have a high overlap, that is if they share many genes.

**Diplotaxodon and deep benthic convergence.** To obtain a quantitative measure of the similarity between and the extent of excess diversity in the Diplotaxodon and deep benthic amino acid sequences, we calculated simple statistics based on the proportions of non-synonymous differences ($p_s$ scores). Intuitively, the similarity score is high if Diplotaxodon and deep benthic jointly have higher $p_s$ than all the others, but are not very different from each other relative to how much diversity there is within Diplotaxodon and deep benthic.

Specifically, the similarity score $s$ is calculated as follows:

\[
 s = \frac{x_{\text{raw}}}{\text{jackknife}_\text{se}(p_s)} = \frac{\text{mean}(x_{\text{raw}})}{\text{jackknife}_\text{se}(p_s)}
\]

where $p_s^D$ is the mean $p_s$ between Diplotaxodon jointly with deep benthic and all the other Lake Malawi species, $p_s^B$ is the mean $p_s$ between Diplotaxodon and deep benthic, and $p_s^W$ is the mean $p_s$ within Diplotaxodon and deep benthic. The jackknife normalization is analogous to the one used for $\Delta_{\text{raw}}$ and the mean ($\text{se}(p_s)$) is subtracted to centre the statistic at zero.

The excess diversity score is high when the mean $p_s$ scores within Diplotaxodon and within deep benthic are high relative to the mean $p_s$ in the rest of the radiation. Specifically, the excess score $ex$ is defined as:

\[
 ex = \left\{ \frac{p_s^D + p_s^B}{2} - p_s^W \right\} / \text{jackknife}_\text{se}(p_s)
\]

where $p_s^D$ is the mean $p_s$ within Diplotaxodon, $p_s^B$ is the mean $p_s$ within deep benthic, and $p_s^W$ is the mean $p_s$ within the rest of the radiation.

**Haplotype trees.** To view the relationship between haplotypes for genes of interest, we translated nucleotide sequences to amino acid sequences and loaded these into Haplotype Viewer (http://www.cbrvtt.ca/~greg/haplotypeviewer). This software requires that a tree is loaded together with the sequences. Therefore, we inferred gene trees using RAxML v7.7.8 with the PROTGAMMA+CAT model of substitution.

**Local allele excess sharing between Diplotaxodon and deep benthic.** We used an extension of the $J_2$ statistic\(^{5}\); this extension is referred to as $J_{2\text{obs}}$ and is a conservative version of the $f$ statistic that is particularly suited for analysis of small genomic windows\(^{27}\). For the gene scores shown in Fig. 6b, we calculated $J_{2\text{obs}}$ (mibuna, deep benthic; Diplotaxodon, N. brichardi) separately for synonymous and non-synonymous mutations in each gene.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Code availability.** The majority of the custom code used in this project is available on Github as a part of the evo package (https://github.com/millanek/evo). All other custom codes are available from the authors upon request.

**Data availability**

All raw sequencing reads have been deposited to the NCBI Short Read Archive: (BioProjects PRJEB1254 and PRJEB15289). Sample accessions are listed in Supplementary Table 4. In addition, we are making whole-genome variant calls in the Variant Call Format (VCF), phylogenetic trees and protein coding sequence alignments, and tables with $f$ statistics available through the Dryad Digital Repository (https://doi.org/10.5061/dryad.7ej6c).

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**References**


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**Author contributions**

E.A.M., G.E.T., M.J.G., M.M. and R.D. devised the study. A.M.T. bred parent-offspring trios and performed geometric morphometric analyses. M.M. performed the DNA extractions. H.S. and M.M. analysed the genetic data. All authors participated in the interpretation of the results. M.M., H.S. and R.D. drafted the manuscript, and all others commented.

**Competing interests**

R.D. declares that he owns stock in Illumina from previous consulting. The authors declare no other competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41559-018-0717-x.

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Our web collection on [statistics for biologists](#) may be useful.

### Software and code

Policy information about [availability of computer code](#).

**Data collection**

| bwa-mem v0.7.10, samtools v1.2, bcftools v1.2, picard-tools v.1.124, GATK v3.3.0, vcftools v0.1.12b, BEAGLE v.4.0 (r1398), shapeit v2 (r790), SPNable tool (http://h3h3.users.sourceforge.net/snpable.shtml), lastz v1.0, Kent UCSC Tools (git://genome-source.cse.ucsc.edu/kent.git; v293_base-36-g9e7d0af), PLINK v1.0.7, eigensofv v5.0.2, RAxML v7.7.8, TreeAnnotator v.2.4.2, BEAST 2.4.2, SNAPP 1.3.0, ETE3 toolkt, PAUP* v4.0a (build 159), ASTRAL v.5.6.1, chrompainter v0.0.4, fineSTRUCTURE v0.0.5, R (and pin particular packages: GeoMorph v3.0.2, topGO v2.26.0, org.Dr.eg.db v3.4.0), bedtools v2.26.0, Haplotype Viewer (http://www.cibiv.at/~greg/haplovieviewer), evo (https://github.com/millanek/evo), modules dstat and tensorfstats of pypopgenan) |

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All raw sequencing reads have been deposited to the NCBI Short Read Archive: (BioProjects PRJEB1254 and PRJEB15289). Sample accessions are listed in Supplementary Table 4. In addition, we are making whole-genome variant calls in the Variant Call Format (VCF), phylogenetic trees and protein coding sequence alignments, and tables with f4 statistics available through the Dryad Digital Repository (see the doi in the "Data availability" section of the manuscript).

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | The goal of our study was to provide primary whole-genome based analysis of the evolutionary diversity of Lake Malawi cichlid fishes. |
| Research sample | Our sample selection provides broad coverage of all the major lineages of the Lake Malawi cichlid radiation: rapidly radiating cichlid tribe Haplochromini. |
| Sampling strategy | We focused on ecomorphological diversity, representing more than half the genera from each major group, rather than obtaining deep coverage of any particular group. |
| Data collection | DNA sequencing was performed by the Wellcome Sanger Institute sequencing core facility |
| Timing and spatial scale | In this study, we sequenced DNA from ethanol preserved finclips that were collected by M.J.Genner and G.F.Turner between 2004 and 2014 from Tanzania and Malawi, in collaboration with the Tanzania Fisheries Research Institute (the MolEcoFish Project) and with the Fisheries Research Unit of the Government of Malawi (various collaborative projects). All of the DNA was extracted and sequenced at the Wellcome Sanger institute between 2012 and 2015. |
| Data exclusions | The analyses used only single nucleotide polymorphism (SNP) data. Larger insertions, deletions, and structural variation were excluded from this study. |
| Reproducibility | All our attempts at replication of analyses were successful, although this was not done in a systematic manner. To assist independent researchers in reproducing and verifying our findings, we provide the SNPs ready to use in a Variant Call Format (VCF) file. |
| Randomization | A number of the reported analyses rely on comparisons between major groups of species, which were determined based on published literature, and correspond to the clustering of the whole genome data. Random assignment was not appropriate. |
| Blinding | During the genomic data analysis, the investigators were aware of the species identity of the genomic sequences analysed (no blinding). |

Did the study involve field work?  Yes  No

Reporting for specific materials, systems and methods
Materials & experimental systems

Methods

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- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Animals and other organisms

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Laboratory animals: Trios were obtained by mating wild-caught fish obtained from Lake Malawi by commercial aquarium fish exporters (Stuart M. Grant Ltd) and exported to UK ornamental fish importers. Adults were quarantined at Bangor University's research aquarium, maintained at ca 25°C in filtered aquaria with a mix of salts added to mimic Lake Malawi water conditions, and fed on standard aquarium fish flake food. Multiple females (5-10) were kept with a single adult male, until a female was seen to be carrying eggs (indicated by her bulging throat and loss of interest in food). Mouthbrooding females were removed to brooding tanks containing shelter for mother in the form of a PVC tube and shelter for the offspring in the form of rocks and artificial plants. When free-swimming fry were observed in the tank (usually after about 3 weeks), the mother was removed and, along with the father, euthanised by anaesthetic (MS-222) overdose. Offspring were reared for 3-6 months, before euthanasia.

Wild animals: The study did not involve wild animals.

Field-collected samples: Finclips were preserved in ethanol for DNA extraction.