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Genomic fingerprints of palaeogeographic history: The tempo and mode of rift tectonics across tropical Africa has shaped the diversification of the killifish genus *Nothobranchius* (Teleostei: Cyprinodontiformes)

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ABSTRACT

This paper reports a phylogeny of the African killifishes (Genus *Nothobranchius*, Order Cyprinodontiformes) informed by five genetic markers (three nuclear, two mitochondrial) of 80 taxa (seven undescribed and 73 of the 92 recognized species). These short-lived annual fishes occupy seasonally wet habitats in central and eastern Africa, and their distribution coincides largely with the East African Rift System (EARS). The fossil dates of sister clades used to constrain a chronometric tree of all sampled *Nothobranchius* recovered the origin of the genus at ~13.27 Mya. It was followed by the radiations of six principal clades through the Neogene. An ancestral area estimation tested competing biogeographical hypotheses to constrain the ancestral origin of the genus to the Nilo-Sudan Ecoregion, which seeded a mid-Miocene dispersal event into the Coastal ecoregion, followed closely (~10 Mya) by dispersals southward across the Mozambique coastal plain into the Limpopo Ecoregion. Extending westwards across the Tanzanian plateau, a pulse of radiations through the Pliocene were associated with dispersals and fragmentation of wetlands across the Kalahari and Uganda Ecoregions. We interpret this congruence of drainage rearrangements with dispersals and cladogenic events of *Nothobranchius* to reflect congruent responses to recurrent uplift and rifting. The coevolution of these freshwater fishes and wetlands is attributed to ultimate control by tectonics, as the EARS extended southwards during the Neogene. Geobiological consilience of the combined evidence supports a tectonic hypothesis for the evolution of *Nothobranchius*.

1. Introduction

Nothobranchius (order Cyprinodontiformes) is a speciose genus of short-lived annual killifishes. These small fishes are recorded in seven of Africa's ichthyological provinces (cf. Lévêque, 1997): Nilo-Sudan, Victoria, Tanganyika, Malawi, East Coast, Zambezi and Congo. This sub-continental distribution of *Nothobranchius* extends from the drainages of the Chari (Chad), across the Nile catchments, and widely across equatorial east Africa (including the offshore islands of Zanzibar and Mafia) and the eastern coastal plain (Mozambique). The western and southern limits

to this distribution are reached in southeast Congo (Katanga), the Zambezi Region (Caprivi, Namibia), and the Pondoland and KwaZulu basins (South Africa), respectively (Fig. 1). All known species are restricted to smaller, sedentary wetlands, strictly not in fast-flowing rivers, nor in flowing non-seasonal rivers or open lakes (Watters, 2014). They include headwater floodplains, seasonally flooded valleys (referred to as dambos in south-central Africa) and marshes skirting lake shores, seepage areas and slow flowing shallow, seasonal stream channels.

The East African Rift System (EARS) dominates the overall range of *Nothobranchius*. The EARS diverges in Ethiopia into two great arcs that

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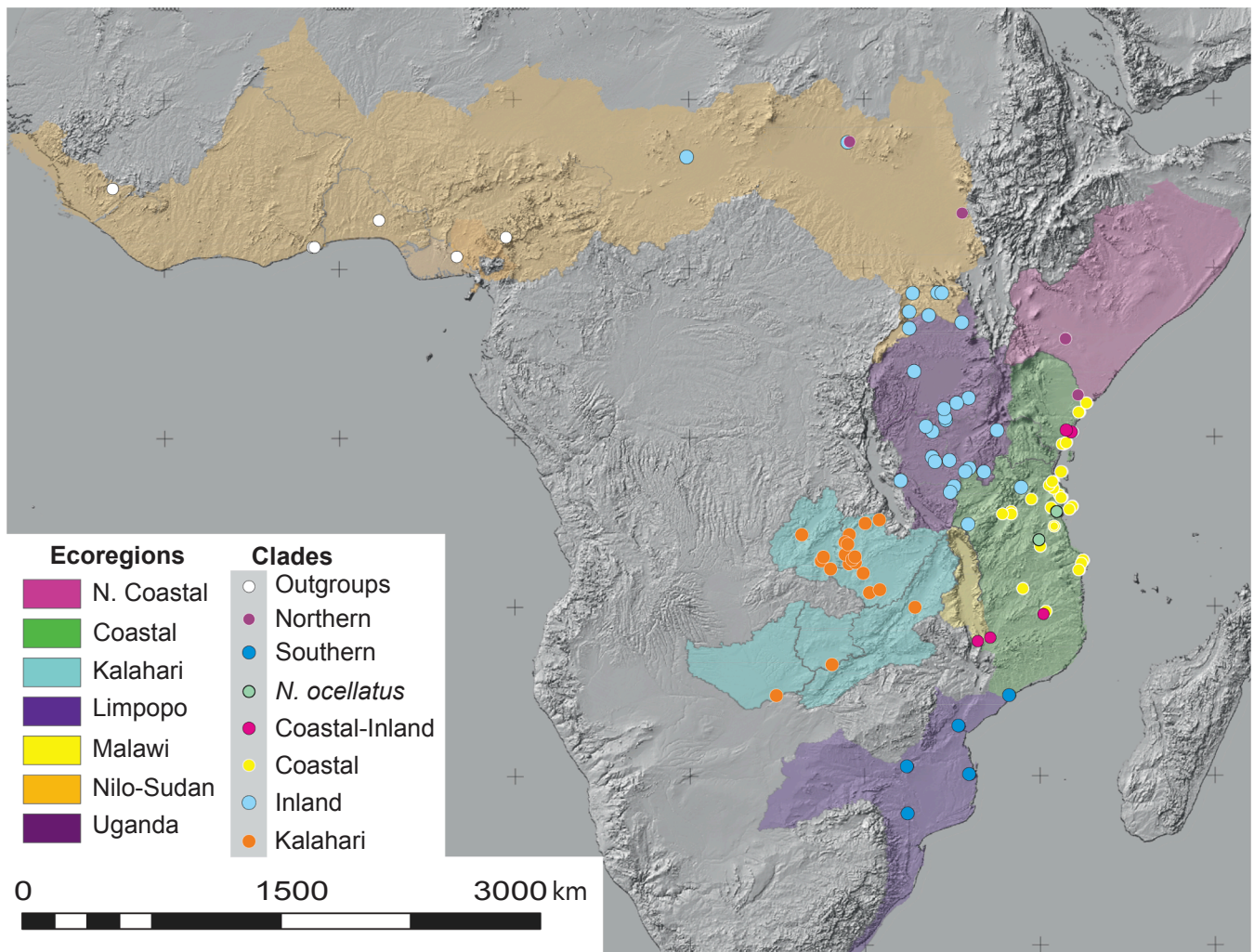


Fig. 1. Map of Sub-Saharan Africa showing the ecoregions (Abell et al., 2008) selected with respect to this study. The localities of *Nothobranchius* and outgroup taxa are shown as coloured dots shaded according to the phylogenetic clade they were retrieved in. The digital surface model (DSM) was derived from the Shuttle Radar Topography Mission (SRTM) (see Flügel et al., 2015).

bisect the subcontinent from north to south (see inset in Fig. 2A). From Lake Turkana, the Gregory Rift extends through Kenya to the North Tanzanian Divergence into the Mid-Tanzanian Rift. Its southeast arm of offshore grabens has shaped the coast (the horsts visible as Mafia, Pemba and Zanzibar), and continues south into the Mozambique Channel. Skirting the Congo Basin, the Western (Albertine) Rift splits into the Malawi Rift and South-West Rift Extension. The latter reaches southern Katanga, continuing across northern Zambia into Botswana (Okavango-Capri). The Malawi Rift continues south through Mozambique, where it is represented in the Urema Trough (Chorowicz, 2005; Steinbruch, 2010; Macgregor, 2015). These principal rifts have been boundaries shaped in their individual structural responses to regional strain of four microplates, notably the counterclockwise rotation of the Victoria microplate (Glerum, et al. 2020). Volcanism has locally accentuated the east African plateau; and together with the tectonically active rifts, the EARS has shaped the regional bimodal topography of “High” Africa in contrast to “Low” west-central Africa (Burke and Gunnell, 2008, see Fig. 1).

The EARS initiated in subcontinental arcs of focal uplift in the Late Oligocene, with distinctive impacts on the drainage of eastern Africa (Key, et al. 2008; Macgregor 2015; Maselli et al., 2020). The grabens and flanking horsts of its rifts testify to a more widespread subcontinental epeirogeny; notably the vast plateaux averaging 1000 m above sea level of southern and east Africa testify to two episodes of uplift, at ~20 Mya and then ~5 Mya (Partridge, 1998, 2010; Partridge et al., 1995; Daly

et al., 2020). This recurring topographic rejuvenation through the Neogene has impacted radically on the drainage of High Africa, and its skirting margins; in tandem with shifting tenures of inland lakes (formed in local sumps), diversions and reversals of rivers have reshaped catchments incrementally. These drainage nets have responded to both diastrophic impacts focused along major fault systems, and the more subtle warping that formed new watersheds.

In places, major rivers have been divided by local impacts of rifting and volcanism. Two examples include (1) lava flows and rifting of the Turkana basin that severed the Omo-Turkana headwaters of the Palaeo-Tana River (L, Fig. 2A, Bruhn et al., 2011); and (2) uplift of the Rukwa-Rungwe Triple Junction (G, Fig. 2A and 2B) isolated the Miocene drainage that formerly linked the Kalahari and Tanzanian plateaux (Cotterill, 2003a; Goodier et al., 2011). In contrast, away from the rift lineaments, the widespread Neogene uplift of Africa resulted in relatively benign changes to river topology. This warping has tended to reshape ancient catchments inherited on ancient erosion surfaces, so these landscapes are still dominated by a senile drainage. Its seasonal dambos and floodplains characterize the watersheds of the Kalahari Plateau and much of the Tanzanian hinterland (Watters, 2009, 2014; Goudie, 2005; Key et al., 2015; Daly et al., 2020; Moore et al., 2020, Fig. 2).

Based on comparative mineralogical analyses of clay-sized fractions of substrate samples, Watters (2009, 2014) presented a comprehensive description and classification of the natural history and habitats of

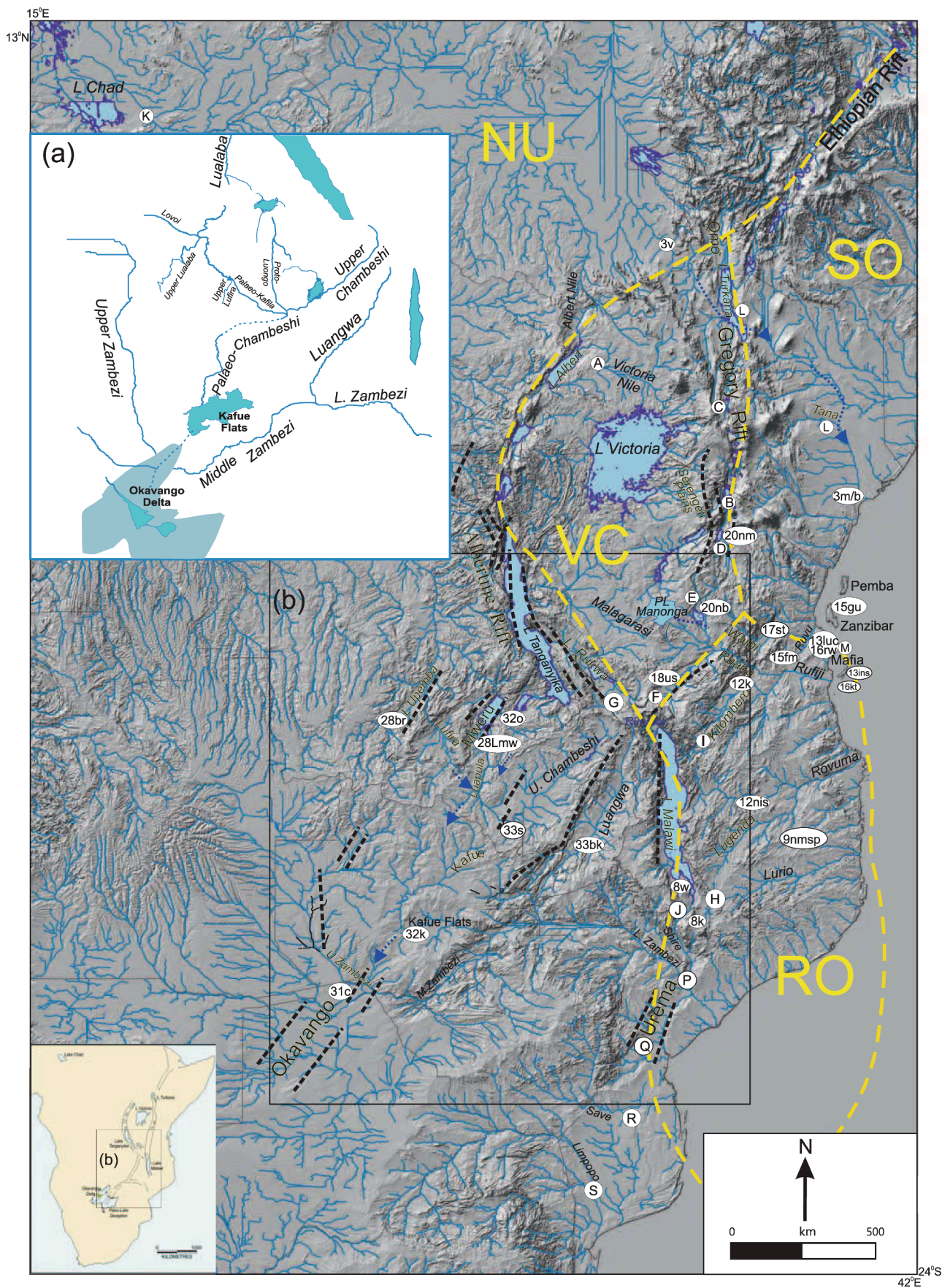


Fig. 2. Fig. 2A (above): A map of central Africa dominated by the plateaux of High Africa, and showing the schematic extent of the east African Rift System (EARS). Details of extant drainage and relief reflect strong controls by the Albertine, Ethiopian and Gregory Rifts and its South-West Extension, and in turn their relationships

with the abutting Nubia, Rovuma, Somalia and Victoria microplates (Dashed red lines, [Glerum et al., 2020](#)). The insert in the bottom left gives an overview of these rifts. The digital surface model (DSM) was derived from the Shuttle Radar Topography Mission (SRTM) (see [Flügel et al., 2015](#)), with drainage represented by the HydroSHEDS digital river database, and the AEON Africa rivers database (see [Goodier et al., 2011](#)). Selected extant and extinct endorheic lakes (palaeo lakes, (PL)) are shown. Numbers and capital letters on the map and the expanded map (Fig. 2B) denote key landforms and formative events in relation to cladogenic and dispersal events, which correspond to the numbers of the clades in [Fig. 4](#) and the letters of dispersals on [Fig. 5](#), respectively, and also correspond to [Fig. 6](#). Key landforms include the separation of the Lake Victoria drainage from the White Nile (A), Lake Natron (B), Tugen Hills (C), Lake Manyara (D), Lake Bahi (E), Usangu Rift (F), Malawi-Rukwa-Rungwe triple Junction (G), rifting of the eastern Malawi horst, which broke and/or reversed flow direction in the Lugenda and Lurio (H), and Ruhuhu (I) rivers, the Shire River (J) draining Lake Malawi, the Chari River flowing into Lake Chad/Palaeo-Lake Chad (K), Palaeo-Tana River (L) that formerly linked precursor drainage of the Upper Nile across the Turkana basin, the Pangani Offshore Rift, including Mafia and Zanzibar horsts (M), and the endorheic Palaeo-Chambeshi drainage system. The schematic (insert a) shows the salient features of the late Neogene Palaeo-Chambeshi drainage system in lower resolution (from region b). The extant drainages of the Chambeshi River draining into the Congo, and the Kafue and Chobe rivers draining into the Zambezi are mapped as solid lines. Antecedent linkages between these drainages of the Palaeo-Chambeshi are indicated by stippled lines. Fig. 2B (below): An expanded map (of region b from [Fig. 2A](#)) with additional detail showing the key palaeo-lakes that existed in discrete tenures during the late Neogene era of the Palaeo-Chambeshi River. Together with turnovers of palaeo-lakes (PL), the breakup of the Palaeo-Chambeshi isolated and/or reversed major tributaries, with widespread impacts on diversification of the Kalahari Clade ([Figs. 3 and 4](#)). These events are represented in the Upper Chambeshi River and principal south-flowing precursors, including the headwaters of the Palaeo-Lufira and Palaeo-Kafila (Na), Proto Luapula (Nb), and Proto-Luongo (Nc) rivers. This late Neogene drainage flowed southwest via the Proto-Kafue (Nd) into a vast Palaeo-Lake (970 m Deception and 945 m Makgadikgadi stands). Breakup of the Proto-Kafue River (Ne) isolated the Palaeo-Kafue River, which thereafter maintained Lake Patrick. Isolation of the Luangwa graben is most plausibly explained by Late Pliocene uplift of the Muchinga Escarpment (O). Principal rivers traversing the Limpopo ecoregion include the: Lower Zambezi (P), Pungwe (Q) Save (R) and Limpopo (S). Palaeoenvironmental data and formative events were collated from F.P.D. Cotterill, (unpublished data), and published sources ([Bloomfield, 1965](#); [Bruhn et al., 2011](#); [Cotterill, 2003a,b, 2005, 2006](#); [Cotterill and de Wit, 2011](#); [Delvaux and Hanon, 2003](#); [Griffin, 2006](#); [Harrison et al., 1996](#); [Key et al., 2015](#); [Le Gall et al., 2004](#); [Lyons et al., 2015](#); [Macgregor, 2015](#); [Macheyeki et al., 2008](#); [Mana et al., 2015](#); [Moore et al., 2012](#); [Partridge, 2010](#); [Scoon, 2018](#); [Talbot and Williams, 2009](#); [Tiercelin and Lezzar, 2002](#); [Van Damme and Pickford, 2003](#); [Watters, 1991](#)). Extents of selected palaeo-lakes were estimated using the contour function in ArcMap 10.1 from extant topography, and are approximate estimates (F.P.D. Cotterill, unpublished data, [Moore et al., 2012](#)). Bold dashed lines denote major faults of principal rift systems (after [Macgregor, 2015](#)); dotted blue arrows approximate former drainage links.

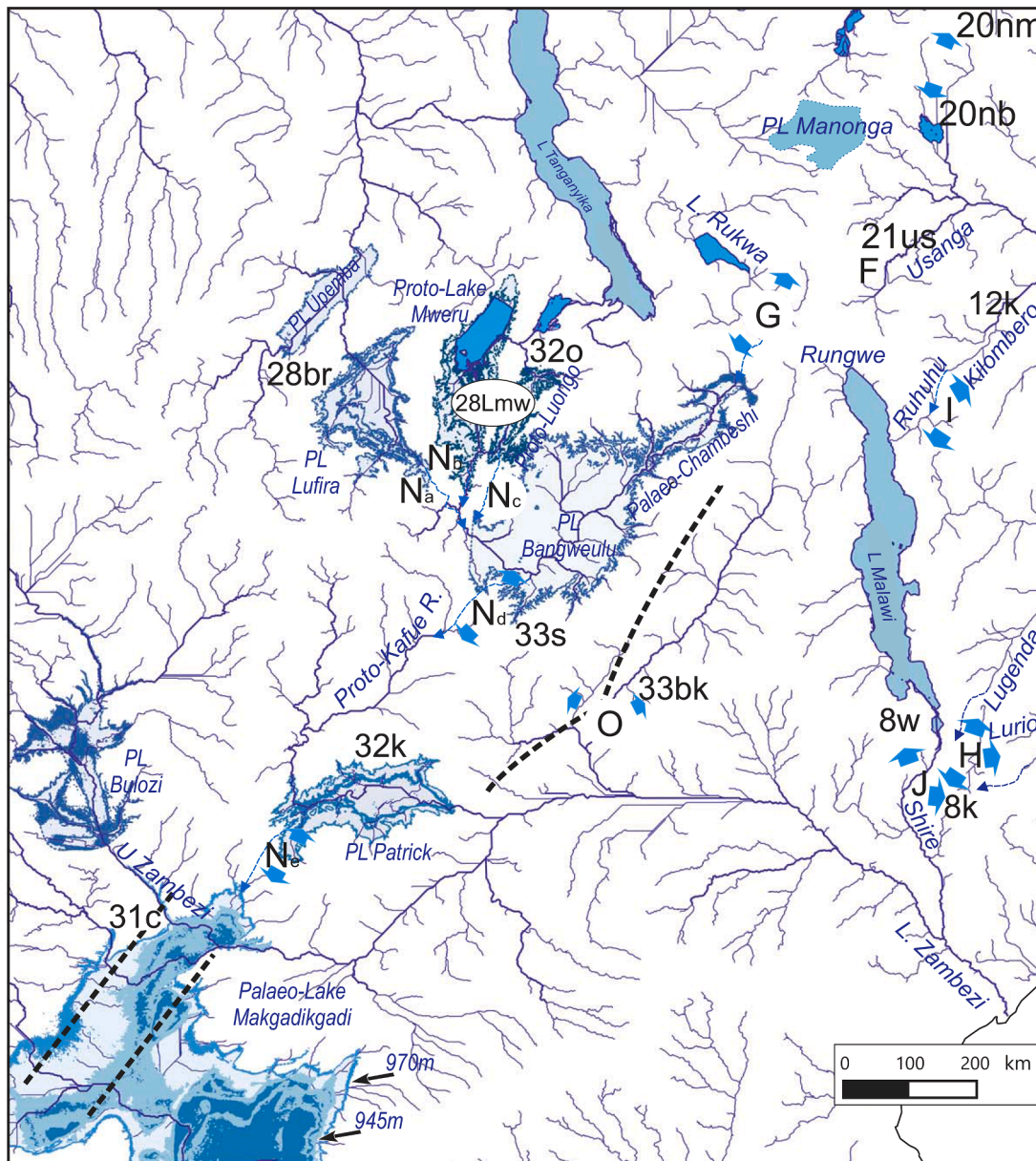


Fig. 2. (continued).

Nothobranchius. These analyses used instrumental techniques to elucidate the physical-chemical character of constituent smectite clays. The hydromorphic properties of these “cracking clay” substrates explain their key role as the primary determinants of the patchy occurrences of these small fishes across tropical landscapes; and, critically, how they persist in arid savannahs despite desiccation of their ephemeral pond habitats. Annualism, a life habit being found more often in the tropics, enables *Nothobranchius* to reproduce in the seasonally dry habitats of African savannahs, and a unique suite of reproductive adaptations defines the annual life histories of *Nothobranchius* (Watters, 2009; Nagy, 2015). Eggs are deposited in the soft mud of the habitat substrate, which has the typical characteristics of a vertisol; critically, the constituent smectite group minerals (mainly montmorillonite) have the capacity to absorb and adsorb water molecules between the layers of their crystal structures, and onto the grain surfaces, respectively. This confers significant swelling properties on the wet clay: which then dries out to crack to significant depth in the soil profile. Through the dry season, the eggs remain viable deep within these cracking clays, which maintain an optimally moist micro-climate in tight proximity with the swelling clay minerals (Watters, 2009; Watters et al., 2019).

As many as three stages of diapause can interrupt the developing embryo within the egg (Peters, 1963; Wourms, 1965, 1972a, 1972b, 1972c; Furness et al., 2015; Pinceel et al., 2015). After hatching at the onset of the wet season, the fry grow rapidly, with sexual maturity attained within three weeks to two months (Markofsky and Matias, 1977; Nagy, 2015; Watters, 2009). It has further been shown by Vrtílek et al. (2018) that *N. furzeri*, the shortest-lived *Nothobranchius* species can, in its natural environment, be sexually mature within two weeks. In combination, this suite of life history traits constrains all these annual fishes to a stenotopic niche. Restricted niche conservatism, in turn, tightly constrains the biogeography of African annual killifishes: expressed in a high fidelity to seasonally desiccated floodplains in tropical savannahs (Watters, 2009, 2014; Cotterill and de Wit, 2011; Cotterill et al., 2016).

Annualism has evolved at least five times and viviparity, another key reproductive adaptation, has evolved at least three times in the Cyprinodontiformes (Helmstetter et al., 2016). Both adaptations explain how these fishes have diversified very successfully in the tropics and into adjacent temperate areas. The Order revealed an accelerated speciation rate in two of the viviparous clades, but not in the annual clades. However, the speciation rate for *Nothobranchius* is likely to be an underestimate, because Helmstetter et al. (2016) analysed 62 species in their estimations, whilst currently 92 species are recognized in the genus. This is reflected in a recent study of 45 predominantly African killifish species, in which the whole genome evolution of representative non-annuals is compared to eight species of *Nothobranchius* (Cui et al., 2019). The authors conclude that the dominance of neutral genetic drift explains how relaxed molecular selection expresses in higher mutation rates associated with evolution of traits (notably short life spans) of annual life histories in arid tropical savannahs. Arguably, the coupling of high mutation rates with neutral genetic drift in short-lived annual populations explains the rapid speciation rates exhibited in *Nothobranchius* (Whitlock and Phillips, 2014). This also explains how relatively short periods of isolation of populations has expressed in the significant genetic divergences documented between, e.g. *N. chochamandai* and *N. sainhousei* (Nagy et al., 2016) and *N. rosenstocki* and *N. cooperi*, respectively (Nagy et al., 2017).

Nothobranchius are weak swimmers: confined within seasonal ponds and pans, and weakly flowing seepage areas and streams. This trait explains their inability to disperse along more strongly flowing rivers, especially upstream against steep gradients. Range expansion in *Nothobranchius* is postulated to occur by means of three possible hypotheses: Firstly, through dispersal of fishes by seasonal flooding along drainage lines referred to as the flood dispersal hypothesis; secondly, by means of the egg dispersal hypothesis by attachment to mammals; and thirdly, by means of egg dispersal by water birds (Watters, 2006, 2009;

Reichard, 2015). The possibility of egg dispersal by mammals or by birds has been rejected or is at best remote (Watters, 2006; Reichard, 2015), because transport by mammals or birds would imply that eggs would have to remain outside water over long periods; and as *Nothobranchius* eggs possess no mechanisms to survive desiccation, even over short periods, they would not survive. Silva et al. (2019), have discovered South American annual killifish (genus *Austrolebias*) eggs in waterfowl faeces and have shown that these eggs can survive a short passage through the alimentary canals of some birds. While these authors invoke this hypothesis of endozoochory as an agent of dispersal, they present no evidence that this mechanism facilitates long-distance dispersal of killifish eggs. Furthermore, it would not only require transport of sufficient viable eggs to establish a new population but, in addition, would require colonization of a seasonal water body having a suitable substrate. We regard this as highly unlikely. Watters (2006) and Reichard (2015) therefore viewed the “flood dispersal hypothesis” as the most likely explanation of how *Nothobranchius* has colonized new habitats.

Dorn et al. (2014) proposed an aridification hypothesis as a cause of speciation of the genus over the Late Cenozoic. Based on their results using the ancestral area estimation method, DIVA, they argued the ancestral area of the genus was the East African coastal region, seeded from West Africa; this explanation ignores ancestral *Nothobranchius* clades in the Nilo-Sudan region. However, Watters (2006, 2009) and Reichard (2015) argued that the major mode of dispersal, and thus cause of diversification has been the reshaping of drainage networks across tropical Africa. This enabled *Nothobranchius* to reach new drainages, where allopatric speciation ensued via vicariance. As argued by Watters (2009) and Cotterill et al. (2016), the evolution of these wetlands has modified distributions of respective *Nothobranchius*, in their twinned responses to uplift and faulting events (explained above). A biogeographic study of selected *Nothobranchius* (i.e. the Southern Clade species *sensu* Dorn et al., 2014) gives significant insights into dispersal and vicariance across the Mozambican coastal plain (Bartáková et al., 2015). The species complexes occurring in this region radiated to the south, where large perennial, west to east flowing rivers are likely persistent dispersal barriers. Their genetic evidence demonstrated episodic southward dispersals across these rivers, which can be explained by the latter’s gentle gradients on the Mozambican plain: resulting in slow flowing rivers, even during extreme floods caused by recurrent cyclones from the Indian Ocean (that impact across coastal Mozambique every 50–100 years). Additionally, this region has been repeatedly reshaped by neotectonics centred on the Urema graben (Steinbruch, 2010) associated with drainage modifications allowing dispersals across the Mozambique coastal plain.

However, just as tectonics may be an agent of speciation, eruptions of at least 615 volcanoes intrinsic to the EARS (Scoon, 2018) have also shaped the evolution of *Nothobranchius* since the Early Miocene. Not only have lava flows modified drainage networks directly, but the predominance of recurrent pyroclastic eruptions of extremely alkaline ash, particularly across the North Tanzanian Divergence Zone of the EARS, would have extirpated *Nothobranchius* populations in shallow wetlands (Altner and Reichenbacher, 2015; Peters et al., 2008, and see Discussion).

Our expanded phylogenetic hypothesis for the genus prompts us to reassess the most likely causes of speciation in *Nothobranchius* across the landscapes of tropical Africa. We examine spatio-temporal patterns of genetic variation within the framework of palaeoenvironmental events (palaeoclimates and drainage changes), to elucidate how vicariance and/or dispersal shaped the evolution of these fishes. A chrono-biogeographical approach (Hunn and Upchurch, 2001; Crisp et al., 2011) structures this study, employing phylogenetic analyses in a cross-disciplinary, geobiological framework (Cotterill and de Wit, 2011; Goodier et al., 2011) leading us to propose a tectonic hypothesis for the diversification of *Nothobranchius* across Eastern Central Africa.

2. Materials and methods

2.1. Taxon sampling

Nothobranchius samples were collected since 1988 by Brian Watters and Béla Nagy during approximately 50 expeditions to wetlands, which discovered many new populations of *Nothobranchius*. In species that occupy comparatively extensive geographical ranges (and therefore potentially constitute species complexes), particular care was taken to sample as many of these populations as possible to verify their affinities. Other selected collectors also contributed particular species for analysis. Specimens of all collections are housed in the ichthyological collections of MRAC, Musée Royal de l'Afrique Centrale, Tervuren and ZSM, Zoologische Staatssammlung, Munich. Wild-caught specimens were used throughout. The annuals *Pronothobranchius seymouri* and *Fundulosoma thieryi*, and non-annual *Aphyosemion bitaeniatum*, *Fundulopanchax gardneri*, *Epiplatys annulatus* and *Aplocheilichthys panchax* were included as outgroups (Supplementary Table 1). In addition, we included a number of species to reconstruct a wider phylogeny of the order to provide secondary constraints to date the *Nothobranchius* phylogeny (Supplementary Table 2).

We paid close attention to the taxonomic veracity of samples. We found that gene sequences we generated from wild-caught vouchers were sometimes quite different to accessions deposited on Genbank. For this reason, we only used gene sequences we generated from tissue samples of reliable provenance, with the exception of gene sequences of some outgroup taxa and *Nothobranchius furzeri*, which were obtained from Genbank (accession numbers are shown in Supplementary Tables 1 and 2). Many *Nothobranchius* sequences on Genbank originate from unreliable aquarium stocks, not vouchered specimens, which obviates independent verification of their provenance.

2.2. DNA extraction, PCR amplification and gene region sequencing

DNA was extracted using a proteinase K and CTAB extraction protocol (Doyle and Doyle, 1987) from either fin clips or muscle tissue of selected specimens (99% ethanol preserved). DNA concentration and purity were determined using a NanoDrop ND1000 spectrophotometer.

Five gene regions were amplified and sequenced from *Nothobranchius* specimens and outgroups. Fragments of three nuclear genes, namely Glycosyltransferase (Glyt), Cardiac alpha (α -myosin heavy chain (MyH6) and Sorting nexin 33, SH3PX3-like domain (SNX33) were amplified using redesigned primers as the published primer sequences (Li et al., 2007) were not found to give consistent amplification in *Nothobranchius*. To solve this problem, the appropriate sequences of these gene regions from the whole genomes of *Nothobranchius furzeri* and the three-spined stickleback (*Gasterosteus aculeatus*) were aligned in Geneious Prime 2019 (<https://www.geneious.com>) and highly conserved regions were identified for primer design. Primers were designed to amplify the complete or nearly complete sequences of two mitochondrial genes, Cytochrome oxidase subunit I (COI) and NADH dehydrogenase 2 (ND2). Designing primers for the amplification of short fragments of mitochondrial genes that would work consistently in all *Nothobranchius* species, as well as selected outgroups, was found to be impossible for these genes, in particular the COI gene, as they showed a high degree of variability especially in the third codon position. Primers were therefore designed to bind to complementary regions in the tRNA genes flanking the COI and ND2 genes, which are much more conserved than regions within the genes themselves. Primers for these regions were designed from an alignment of complete mitochondrial genomes of *Nothobranchius furzeri* (NC011814), *Kryptolebias marmoratus* (KT893707) and *Austrolebias charrua* (KP718940). Where ambiguities were observed in the consensus alignment, primer bases were designed to be identical/complimentary to the *Nothobranchius furzeri* genome. We identified a relatively conserved region approximately in the middle of the COI gene, wherein two internal sequencing primers were designed

(the reverse primer is the reversed and complemented sequence of the forward primer). All the primers used for this study are listed in Supplementary Table 3.

Polymerase chain reaction (PCR) amplifications of all gene regions were performed on an Applied Biosystems Veriti 96-well Thermal Cycler in 25 μ l volumes. PCR mixtures consisted of 15.9 μ l ddH₂O; 2.5 μ l MgCl₂ (25 mM); 2.5 μ l 10X JMR-455 PCR buffer (Southern Cross Biotechnology, Cape Town, RSA); 0.1 μ l Supertherm Taq (5 U μ l⁻¹, Southern Cross Biotechnology, Cape Town, RSA); 1 μ l of a 5 mM dNTP solution (KAPA Biosystems, Cape Town, RSA) 0.25 μ l of the respective specific forward and reverse primers for each gene region respectively (20 μ M), 0.25 μ l BSA (4 μ g μ l⁻¹); 1 μ l of template DNA solution (50–100 ng μ l⁻¹) and DMSO (1.25 μ l).

PCR amplifications of the different gene regions were performed as follows: COI: 80 °C for 5 min; 35 cycles of 95 °C, 1 min, 58 °C, 1 min, 72 °C; 5 min 72 °C for 5 min. ND2: 80 °C for 5 min; 35 cycles of 95 °C, 1 min, 58 °C, 1 min, 72 °C; 5 min 65 °C for 5 min. MyH6 and Glyt: 94 °C for 5 min; 35 cycles of 94 °C, 30 sec, 62 °C, 50 sec, 72 °C; 1 min; 72 °C for 7 min. SNX33: 94 °C for 5 min; 35 cycles of 94 °C, 30 sec, 60 °C, 50 sec, 72 °C, 1 min; 72 °C for 7 min. In all amplifications, ramp rates of 0.53 °C/sec were used. PCR products were sequenced in the forward and reverse direction with the primers as specified above at Stellenbosch University's Central Analytical Facility. Forward and Reverse sequencing chromatograms were super-imposed and base calling corrected in ChromasPro v2.1.8. Consensus sequences were then imported and aligned in Geneious using the Muscle v3.8.31 algorithm (Katoh and Standley, 2013). All codon positions were checked and minor manual corrections in the alignments were made to ensure correct alignment of codon positions, in especially the COI and ND2 genes.

We used the mitochondrial ND2 and COI sequences to confirm homology and sequence similarity within species and populations. Based on those results, we sequenced the conserved nuclear regions in a few populations within each species.

2.3. Sequence and phylogenetic analysis

To establish a phylogeny for the genus *Nothobranchius* we used the sequences of two mitochondrial (ND2 and COI) and three nuclear (MyH6, Glyt and SNX33) loci. Sequence alignments of the mitochondrial ND2 and COI genes were codon partitioned. Codon position saturation was checked with DAMBE 7.0.28 (Xia, 2018) using the transition and transversion versus divergence function. Models of evolution of each of the five gene region alignments were determined prior to analysis using jModelTest v.2.1.6 (Darrriba et al., 2012) on the CIPRES Science Gateway (Miller et al., 2010). Combined codon partitioned alignments of the ND2 and COI genes and the gene partitioned nuclear genes (MyH6, Glyt and SNX33) were used for phylogenetic analysis using maximum likelihood (ML, RAxML 8.0, Stamatakis, 2014) and Bayesian (BI, MrBayes 3.2.6, Ronquist and Huelsenbeck, 2003) approaches on CIPRES. Three heated chains and one cold chain were employed in the Bayesian analyses, with runs initiated from random trees. Two independent runs were conducted with 20 million generations per run; and trees and parameters were sampled every 500 generations. Convergence was checked using Tracer 1.5 (Rambaut et al., 2017). For each run, the first 10% of sampled trees were discarded as burn-in. Majority-rule consensus trees were generated from the individual trees retrieved from the Bayesian analyses. Tree files were imported into Figtree (Rambaut, 2009) for tree drawing (<http://ree.bio.ed.ac.uk/software/figtree/>).

For the dating approach we generated a second alignment matrix based on the sequences of the MyH6, Glyt and SNX33 gene regions of a number of outgroup taxa (Genbank, Supplementary Table 2). It includes two outgroup species in the respective orders most closely related to the order Cyprinodontiformes, i.e. *Menidia beryllina* in the Atheriniformes and *Polycentrus schomburgkii* in the Polycentridae. A number of additional genera in two of the three families in suborder Aplocheiloidei in the Cyprinodontiformes were included. In the Aplocheiloidei, the

genera *Pachypanchax* from Madagascar and the Seychelles, and *Aplocheilichthys* from India in the Aplocheiloidei, were included as they have been found to be sister to the African Nothobranchiidae (Pohl et al., 2015; Helmstetter et al., 2016). In addition, we included the two African Nothobranchiidae genera most closely related to *Nothobranchius* (*Pro-nothobranchius* and *Fundulosoma*) and the more distantly related *Fundulopanchax*, *Aphyosemion*, *Fenerbahce* and *Epiplatys*. The sequences of 19 outgroup taxa from the above groups were aligned using MUSCLE v3.8.31 (Edgar, 2004a, 2004b) and manually refined with the sequences of ten key selected *Nothobranchius* taxa which we had generated representing all clades (see Supplementary Table 2). The datasets were aligned as described above, combined in Geneious and partition models determined using jmodeltest.

2.4. Molecular clock analysis

To date the *Nothobranchius* phylogeny we used the second alignment matrix of three conserved nuclear genes of taxa in the Cyprinodontiformes and its most closely related families, i.e. the Atheriniformes and the Polycentridae mentioned above. Seven selected dated nodes generated for bony fishes (Near et al., 2013; as updated by Sanciango et al., 2016 and implemented in TimeTree (Kumar et al., 2017)) were used to calibrate the tree using a normal distribution with a standard deviation of 10%. The constrained nodes included the *Polycentrus* stem age (96.5 million years ago (Mya)), the most recent common ancestor (MRCA) of the Atheriniformes and the Cyprinodontiformes (77.4 Mya), the MRCA of the Cyprinodontiformes (69.3 Mya), the MRCA of Fundulidae, Cyprinodontidae and the Poeciliidae (47.2 Mya), and the MRCA's of the genera *Fundulus* and *Lucania* (22.5 Mya), *Cyprinodon* and *Jordanella* (15.3 Mya) and *Poeciliopsis* and *Gambusia* (14.2 Mya) respectively. Dating of these basal nodes in the Cyprinodontiformes and its most closely related families was performed using BEAST (v. 1.8.4; Drummond and Rambaut, 2007) on CIPRES with uncorrelated log-normal clock settings and the birth-death model over 20 million generations.

The ages of six basal nodes (mean age with 95% HPD values) in the Cyprinodontiformes generated by this higher level analysis and the five gene *Nothobranchius* alignment matrix was then used in a second BEAST analysis to calibrate the entire *Nothobranchius* phylogeny using the same dating settings as above. This alignment was reduced where prior phylogenetic analyses showed monophyletic species (with one exception, the Lake Mweru complex) in order to allow the ancestral area estimation on the BEAST generated phylogeny.

2.5. Ancestral area estimation

In the ancestral area estimation, the localities of taxa included in the five gene phylogenetic analysis were classified on a map of African freshwater ecoregions (derived from Thieme et al. (2005) and Abell et al. (2008)). Constrained to specific drainages in which *Nothobranchius* occur, we coded each locality datum to a respective ecoregion. Although our regional delimitation resembles that of Dorn et al. (2014), we have restricted the total number of ancestral areas to eight. Two (Nilo-Sudan and Coastal) coincide with the ichthyological provinces of Lévêque (1997) and we have further subdivided the Inland region into the Uganda and Kalahari regions. The areas are defined and motivated as follows (ecoregion nomenclature according to Abell et al. (2008)):

1. India: The most basal outgroup, *Aplocheilichthys*, occurs in India.
2. Nilo-Sudan: Outgroups more closely related to *Nothobranchius* are distributed across a number of ecoregions in western and northern Africa, but *Nothobranchius* is only found in ecoregion 522.
3. Northern Coastal: *Nothobranchius* species found in ecoregion 531.
4. Coastal (Gregory Rift): Species found in the ecoregions 567, 568 and 564 were included in the Coastal area and these include the islands of Mafia and Zanzibar.

5. Limpopo: This region is restricted to part of the Zambezi ichthyological province, i.e. ecoregion 576.
6. Uganda (including Albertine Rift): Species occurring in the areas around and south of Lake Victoria in ecoregions 521, 543, 565 and 566 were coded to occur in the Uganda region.
7. Kalahari (Southwest Rift Extension): All species occurring in the ecoregions 544, 545, 556, 557 and 588.
8. Malawi (Upper Shire River): The ecoregion 559 was coded separately as the Malawi region because this drainage basin is separated from the coastal areas. Only *N. wattersi* occurs in this region.

We performed the ancestral area estimation using BioGeoBEARS v1.1.1 (Matzke, 2018) implemented in R (R Core Team, 2013, R x64 v3.5.2) on the maximum clade credibility *Nothobranchius* phylogeny generated with Tree Annotator 1.8.4 from the BEAST package. The BioGeoBEARS method implements three likelihood-based models: Dispersal-Extinction-Cladogenesis (DEC; Ree and Smith, 2008), the likelihood version of dispersal–vicariance (DIVA; Ronquist, 1997; herein DIVALIKE), and the likelihood version of BayArea model (Landis et al., 2013; herein BAYAREALIKE). The “J” parameter (founder event/jump speciation) allowing descendant lineages to have a different and disjunct area (long distance dispersal) from the direct ancestor was added (Matzke, 2013). In order not to violate the assumptions on which these analyses are based, the number of populations per species or per operational taxonomic unit, where species have not been described, was reduced to one in R. Only four species were coded to more than one ancestral area: *N. microlepis*, *N. jubbi* and *N. patrizii* (Northern Coastal and Coastal), and *N. ugandensis* (Nilo-Sudan and Uganda). A total of six models were compared in an unconstrained analysis. Max area was set to eight, reflecting our defined regions.

2.6. Mapping of tectonic and cladogenic events

As described above, a suite of life history traits delimits the stenotopy of all extant *Nothobranchius*. Collectively, this evolutionary constraint restricts the ranges of all populations to within low-energy wetlands (floodplains and dambos) having substrates with a significant smectite clay component (Watters, 2009). If a peripatric event seeded a killifish population in any other habitat, the absence of key breeding substrate would have doomed such founders to rapid extinction. Thus, as demonstrated in previous studies of stenotopic freshwater fishes (Cotterill and de Wit, 2011; Goodier et al., 2011), we can invoke climatic and/or tectonic events as candidate biogeographical controls: notably where epeirogenic axes formed watersheds, and fault systems disrupted drainage lines. Mesoscale landforms formed during these formative events continue to exercise persisting controls on drainage patterns, and dependent populations of extant fishes. Detailed review of the earth science literature identified candidate aquatic landforms and/or relief as relevant biogeographic controls of killifishes. The geochronology of respective physiographic controls have been dated (correlated to discrete episodes of tectonism), and mapped accurately by investigating geologists. A total of 19 tectonic events (formative events) were singled out as relevant controls over the dispersal and speciation of *Nothobranchius* species. These are mapped in QGIS 3.4 (labelled A to S, Fig. 2A). Key tectonic events are discussed below, but beyond citing respective authorities, their detailed description is beyond the scope of this paper.

As represented from the combined inventory effort, the majority of the *Nothobranchius* species have very restricted distributions, and of the total 92 known distributions, only a few (e.g. *N. melanospilus*, *N. furzeri*, *N. orthonotus*, *N. pienaar*) occur over two or more adjacent drainages. It must also be emphasized that a species is always confined in smaller pools, never in large lakes and river channels; that is, ranges are confined by tight stenotopy. No extant species is known to be distributed

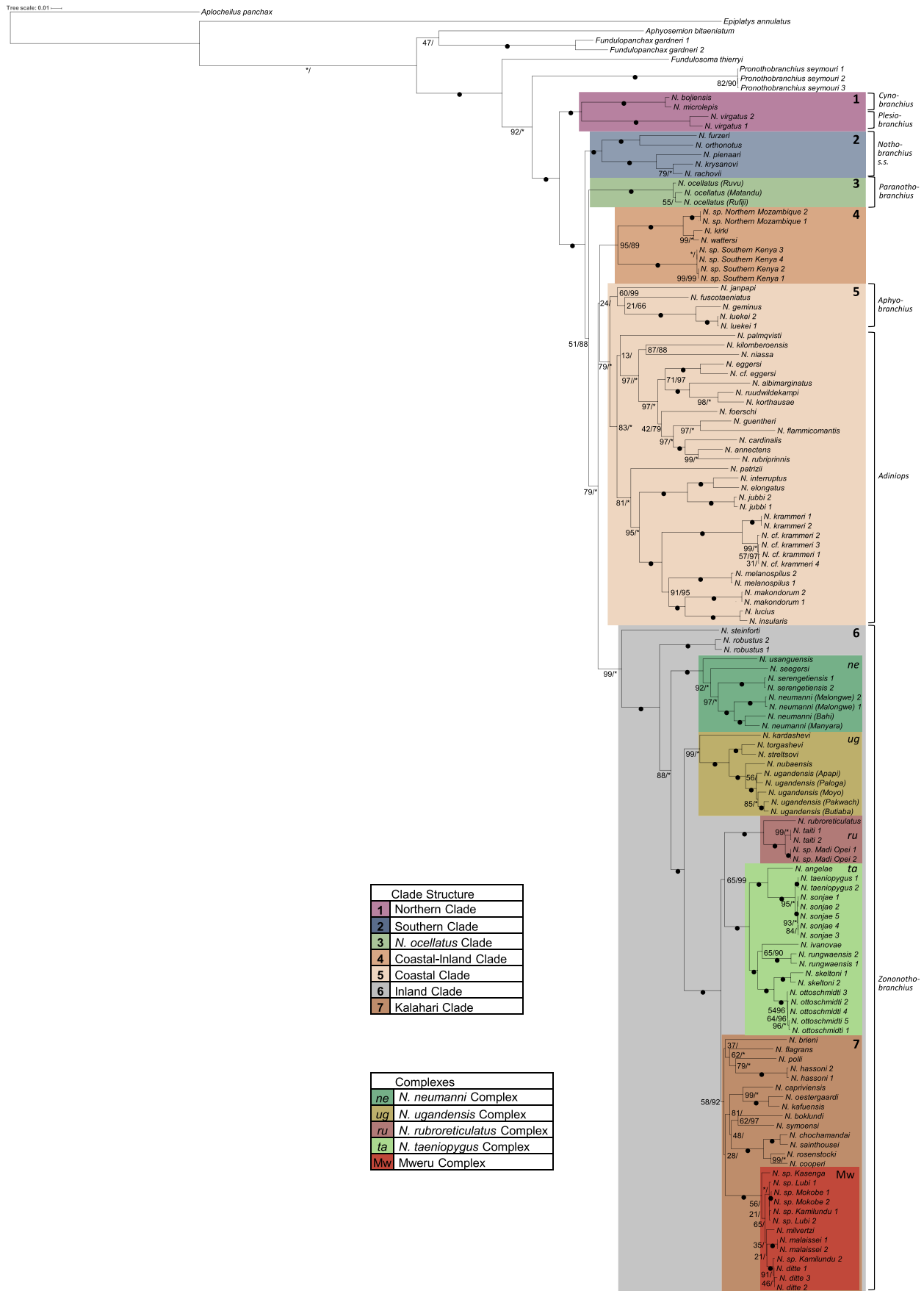


Fig. 3. Fig. 3A (above): Maximum likelihood five gene phylogeny with maximum likelihood bootstrap values and Bayesian posterior probabilities indicated on the nodes. Nodal supports of maximum likelihood bootstrap values of 100%, and Bayesian posterior probabilities of 1, are indicated with a solid dot; nodes where one of either values is 100% or 1 are indicated with a star. The actual value is indicated on nodes of lower support. Retrieved clades and complexes are shaded, numbered, and distinguished by respective letters. For comparative purposes the same colour coding is used in Fig. 4. Fig. 3B (below): Photographs of selected male specimens of species representative of the phylogenetic clades, subgenera and species complexes within the genus *Nothobranchius*. **Northern Clade (1):** A - *N. microlepis*, B - *N. virgatus*; **Southern Clade (2):** C - *N. orthonotus*, D - *N. rachovii*, E - *N. furzeri*; ***N. ocellatus* Clade (3):** F - *N. ocellatus*; **Coastal-Inland Clade (4):** G - *N. kirki*; **Coastal Clade (5):** H - *N. luekei*, I - *N. fuscotaeniatus*, J - *N. melanospilus*, K - *N. eggersi*, L - *N. jubbi*, M - *N. kilomberoensis*; **Inland Clade (6):** N - *N. neumanni*, O - *N. ugandensis* (Blue/yellow phenotype), P - *N. ugandensis* (red phenotype), Q - *N. nubaensis*, R - *N. rubroreticulatus*, S - *N. angelae*; **Kalahari Clade (7):** T - *N. kafuensis*, U - *N. malaissei*. All photographs by Brian Watters, except A and U by Béla Nagy.



Fig. 3. (continued).

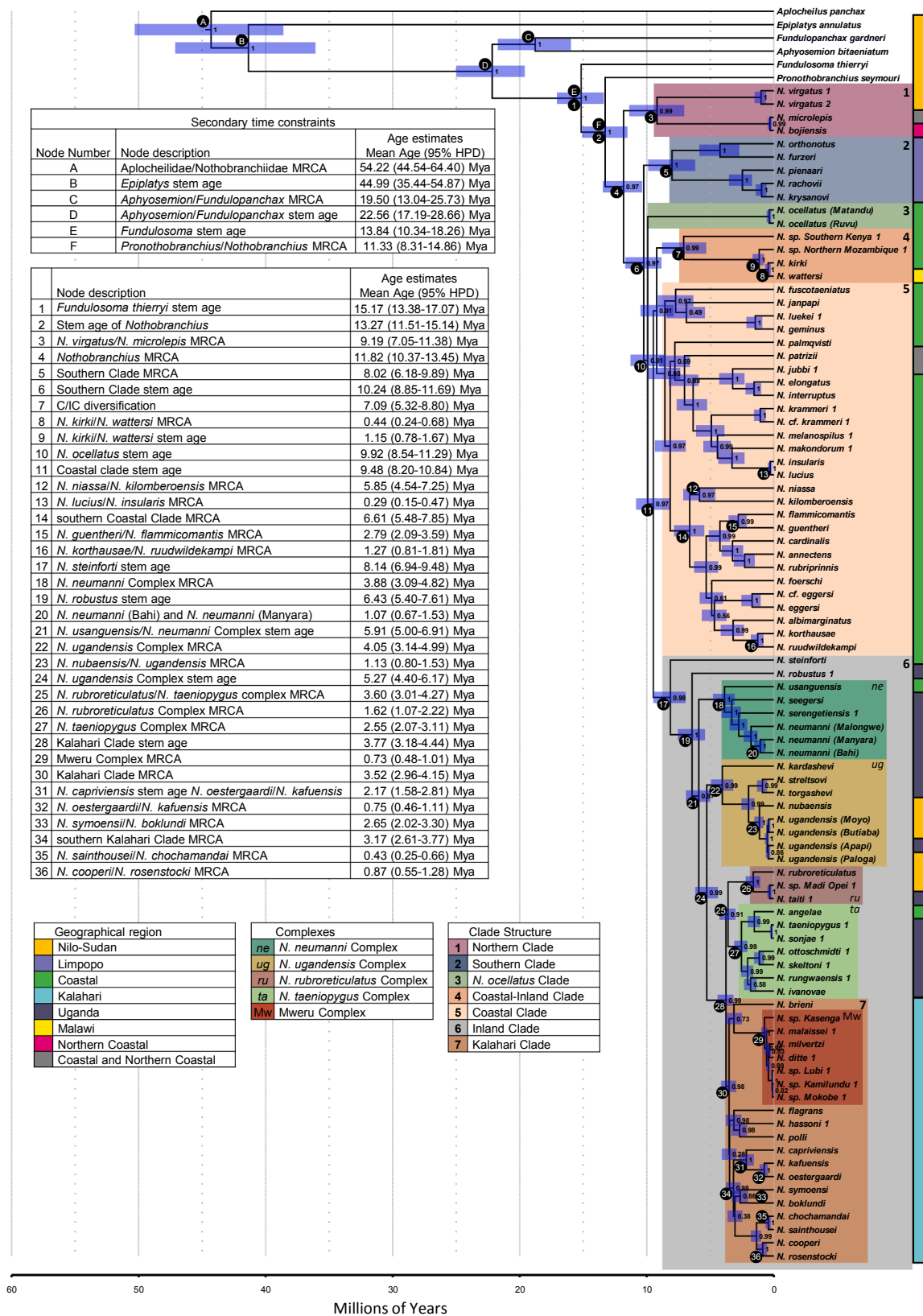


Fig. 4. Maximum clade credibility tree inferred from BEAST based on the combined datasets of five DNA regions to estimate the ages of the *Nothobranchius* radiation. Node support is indicated with posterior probability values, and the bars around node ages indicate 95% highest posterior density intervals. Constrained node ages are indicated in the upper inserted table (A-F). The clades and complexes retrieved are numbered 1–36, with respective age estimates indicated (lower inserted table). Colour coding of clades corresponds to Fig. 3A, and the node numbers in the inserted table correspond to mapped numbered localities (Fig. 2A and 2B) for inferred cladogenic events, based on respective geological evidence. Respective geographical regions are colour coded (bar, chronogram right – indexed lower left) in correspondence to colour coding in Fig. 5.

across the whole range of the genus, nor does a species extend over two, nor even one whole ecoregion. Many species are restricted only to a few pools within a very small area. Based on the stenotopic ecology of the genus, we argue that known localities provide a reliable estimate of a species' distribution within the limits of mapped aquatic landforms and the bounding catchment of the drainage net. Thus, the collective localities of the species, mapped at the large scale of Fig. 1, provide a robust approximation of species' ranges (even for species occupying larger ranges). At the finer scale (Fig. 2A, and Fig. 2B in particular), the single collection locality per species delimits respective distributions to within the physiographic confines of that wetland and/or drainage basin. Obviously, as approximations, these indicators do not outline exact ranges of species. The localities that indicate the representative species are indicated as encircled numbers in Fig. 2A and 2B, e.g. the position indicated by the encircled number 17 indicates the locality where *N. steinforti* was collected. Cladogenic events dating key speciation events in the genus are listed in the accompanying table in Fig. 4. Where such cladogenic events give rise to two sister species or sister groups, the localities of these sister species or groups are indicated by the encircled number of the clade with a small letter indicating the species they represent, e.g. in the case of the cladogenic event 3, they are indicated as 3v, representing the locality of *N. virgatus* and 3m/b indicating the locality of both *N. microlepis* and *N. bojiensis* (Fig. 2A).

3. Results

3.1. Taxon sampling, DNA extraction, PCR amplification and gene region sequencing

Our extensive taxon sampling for this study included 73 of the 92 *Nothobranchius* species currently recognized, plus 7 taxa with an affiliation yet to be determined, see Supplementary Table 1. The localities of respective collections are indicated in Fig. 1. Co-ordinate data are not provided for conservation reasons, but these can be supplied to accredited conservation authorities. In all populations more than one individual was used for DNA isolation, and a number of populations were analyzed of some species.

Our genotyping of seven *Nothobranchius* collections within the ranges of widely distributed species has discovered previously unrecognized lineages, which are phylogenetically distinct. Further evaluations of their respective morphological characteristics are in process to confirm if particular lineages represent distinct species (namely *N. sp.* "Southern Kenya", *N. sp.* "Northern Mozambique", *N. cf. eggersi*, *N. cf. krammeri*, *N. sp. Madi Opei*, *N. sp. Kasenga* and a further species in the Mweru complex). Following completion of the present study, an additional seven species have been formally described (Nagy et al., 2020; Watters et al., 2020), and their genotypes support the phylogenetic findings of this paper (van der Merwe et al., unpublished data).

Although DNA extraction was generally successful, partial degradation of DNA in isolates from some older specimens made PCR amplification problematic. This necessitated the amplification of larger genes in shorter fragments (see Supplementary Table 3 for primer sequences). The primers designed for the amplification of our five loci (Supplementary Table 3) were successful for the amplification of these regions in almost all *Nothobranchius* species including outgroups.

3.2. Sequence and phylogenetic analysis

The characteristics of the COI, ND2, Glyt, MyH6 and SNX33 genes are shown in Supplementary Table 1.

The best-fitting model for the maximum likelihood (ML) analysis and the Bayesian inference (BI) of all five gene regions was determined to be the GTR + I + G model. The phylogeny generated from the combined five gene matrix by the maximum likelihood analysis is shown in Fig. 3A with maximum likelihood bootstrap values and Bayesian posterior probabilities. The Bayesian analysis of the combined five gene matrix

was almost identical, with minor soft incongruences (nodes with $\leq 70\%$ bootstrap support) in the Inland Clade (Supplementary Fig. 1).

Partitioning of the ND2 and COI genes into first, second and third codon position showed that the third codon position of the ND2 showed partial saturation and the COI gene was saturated (Supplementary Fig. 2). Phylogenetic analyses were run with or without the saturated third base position of the COI and ND2 gene, and it was found that the generated phylogenies showed the same basal resolution and clade support values; but terminal branches and relationships of terminals were less well supported if the third base position of the COI gene was excluded (Supplementary Fig. 3), which is in concordance with results from Nagy et al. (2016) and Watters et al. (2019). For this reason, the third codon position data was included in the analyses. The sequences of the more conserved nuclear genes contributed to the basal resolution of the phylogeny, albeit that some basal nodes remained poorly supported based on lower maximum likelihood bootstrap values; Bayesian posterior probabilities indicated these nodes to be supported.

The phylogeny retrieved a strongly supported monophyletic *Nothobranchius* in the ML and BI analyses within which there were a number of strongly supported clades (Fig. 3A). Our more thorough taxon sampling confers much greater clarity to the relationships – and evolutionary affinities in the genus *Nothobranchius* (Fig. 3B). We retrieved six clades, five of which we have given the same names as those of Dorn et al. (2014), i.e. the Northern Clade, the Southern Clade, the Coastal Clade, the Inland Clade as well as the *Nothobranchius ocellatus* Clade. Furthermore, our phylogeny shows a sixth clade, not found by Dorn et al. (2014), which we have called the Coastal-Inland Clade in accordance with where its members occur. All clades were retrieved with high bootstrap support (with the exception of the *Nothobranchius ocellatus* Clade).

As the phylogenetic affinities and relationships of species are self-evident in Fig. 3A, only selected key features of the phylogeny will be discussed hereafter: The Coastal-Inland Clade contains two as yet undescribed species, one from southern coastal Kenya, *N. sp.* "Southern Kenya", sister to the second, *N. sp.* "Northern Mozambique" from northern coastal Mozambique and *N. kirki* and *N. wattersi* from inland Malawi. Its overall range coincides largely with the Rovuma microplate, and south of the Somalia microplate (Fig. 2A). The Inland clade identifies certain ancestral lineages such as *N. steinforti* and *N. robustus*, a clade consisting of *N. usanguensis*, *N. seegersi*, *N. serengetiensis* and *N. neumanni* found southwest, south and southeast of Lake Victoria (the *N. neumanni* Complex), and a cluster of clades containing *N. nubaensis*, *N. ugandensis*, as well as species occurring south of Lake Victoria (*N. kardashevi*, *N. torgashevi*, and *N. streltsovi*, the *N. ugandensis* Complex). Branching from this deepest node is the second major clade, indicating a subsequent radiation of species (the *N. rubroreticulatus* Complex). It contains *N. rubroreticulatus* which is found in ponds in the Chari River system draining into Lake Chad, whilst its sister species, *N. sp. Madi Opei* and *N. taiti* are found in the Nile drainage in northern Uganda. This geographically discrete radiation has occurred north of the Victoria microplate, which confines all other known species of the Inland clade (Figs. 1 and 2A and 2B). The morphologically similar species, *N. bellemansi*, from the southern part of the Upper Nile basin (in Sudan), is most probably a member of this clade (Valdesalici, 2014). The recently reviewed *N. taeniopygus* Complex consisting of seven species occurring south of Lake Victoria (Watters et al., 2019), is further retrieved as a separate clade.

All species found in the Katanga Province of the Congo, Zambia and the Zambezi Region of Namibia comprise a distinct sister clade (the Kalahari Clade). Many species appear in unresolved basal positions in this clade, such as *N. brieni* from the drainages of the Upemba River, *N. hassoni*, *N. flagrans* and *N. polli* from the Lufira River and *N. boklundi* from the Luangwa River. A clade containing *N. oestergaardi*, *N. kafuensis* and *N. capriiviensis* was also retrieved. The clade consisting of *N. sainthousei*, *N. chochamandai*, *N. rosenstocki* and *N. cooperi* represents species that evolved in different headwaters of the Luapula River system. A further clade includes *N. malaissei*, the recently described *N. milvertzi* (Nagy, 2014) and *N. ditte* (Nagy, 2018), and related populations. These

Table 1

The BioGeoBEARS comparison of the fit of different models of biogeographical range evolution and model specific estimates for different parameters (d = dispersal, e = extinction, j = weight of jump dispersal (founder speciation)).

Model	Model parameter no.	LnL	d	e	j	AIC	AIC weight
DEC	2	-92.885	3.01×10^{-3}	1.00×10^{-12}	0	189.8	2.04×10^{-2}
DEC + j	3	-88.023	1.53×10^{-3}	1.00×10^{-12}	1.06×10^{-2}	182	9.68×10^{-1}
DIVALIKE	2	-102.24	4.52×10^{-3}	1.00×10^{-12}	0	208.5	1.8×10^{-6}
DIVALIKE + j	3	-92.487	1.50×10^{-3}	1.00×10^{-12}	1.10×10^{-2}	191	1.11×10^{-2}
BAYAREALIKE	2	-140.91	4.01×10^{-3}	4.95×10^{-2}	0	285.8	2.80×10^{-23}
BAYAREALIKE + j	3	-96.735	1.14×10^{-3}	1.00×10^{-7}	1.30×10^{-2}	199.5	1.60×10^{-4}

have diverged in isolated wetlands fringing the present-day Lake Mweru (the Mweru Complex).

3.3. Molecular clock dating

The maximum clade credibility tree of the order Cyprinodontiformes, and two species in the outgroups in the Atheriniformes and the Polycentridae respectively, agrees with that of Near et al. (2013), Pohl et al. (2015) and Helmstetter et al. (2016), although we included many other close relatives of *Nothobranchius*. The estimated dates of nodes are indicated in tabular form in Supplementary Fig. 4.

The ages of six of those nodes were used for the calibration of the *Nothobranchius* phylogeny (Fig. 4). We recovered a nested sequence of radiations that have shaped the extant diversity of *Nothobranchius*. A tabulated summary of the divergence age estimations for key nodes is shown as mean ages with 95% HPD values (Fig. 4). The initial radiation of the Northern Clade dates to 11.82 [10.37–13.45] Mya (node 4), followed by the radiations of the Southern, *N. ocellatus*, Coastal-Inland and Coastal Clades between 10.24 [8.85–11.69] Mya (node 6) and 9.48 [8.20–10.84] Mya (node 11). The Inland Clade at 8.14 [6.94–9.48] Mya (node 17) in turn radiated into a number of complexes, and the Kalahari Clade which has diversified across central Africa since 3.52 [2.96–4.15] Mya (node 30).

The divergence of *Nothobranchius rubroreticulatus* from *N. sp.* Madi Opei and *N. taiti* at 1.62 [1.07–2.22] Mya (node 26) dates the singular dispersal event from the Nile drainage into the Sahelian drainage network. Three pairs of taxa were sampled deliberately to account for linkages between island and mainland species: *N. lucius*, mainland versus *N. insularis*, Mafia Island; *N. flammicomantis*, mainland, and *N. guentheri*, Zanzibar Island; and *N. ruudwildekampi*, mainland, and *N. korthausae* Mafia Island. Their divergences date to 0.29 [0.15–0.47] Mya (node 13), 2.79 [2.09–3.59] Mya (node 15) and 1.27 [0.81–1.81] Mya (node 16), respectively.

3.4. Ancestral area estimation (AAE)

Among the six candidate models, the AAE in BioGeoBEARS indicates lower AIC values for three parameters in comparison to two parameters (Table 1); this indicates jump speciation has been an important process in range expansion in *Nothobranchius*. The BioGeoBEARS analyses recovered DEC + j as the best-fit biogeographical model (AIC = 182). We present the AAE of BioGeoBEARS under the DEC + j model in Fig. 5 and the AAE under the DEC model in Supplementary Fig. 5.

Since the early Neogene, *Nothobranchius* colonized new habitats through a sequence of dispersal events which were each followed by subsequent speciation events. The AAE in BioGeoBEARS reconstructs the ancestral area of the genus to be the Nilo-Sudan region (Fig. 5). From there, one dispersal south to the Coastal region of East Africa occurred at 10.24 [8.85–11.69] Mya (Fig. 4; node 6) and another dispersal to the Coastal region at 9.19 [7.05–11.38] Mya (node 3) with a range extension into the Northern Coastal Region at 0.3 [0.15–0.5] Mya. Malawi was colonized as a single event from the Coastal Region at 0.44 [0.24–0.48] Mya (node 8). The Limpopo and Uganda regions were colonized from the Coastal Region around 9.48 [8.20–10.84] Mya (node 10) and 8.14 [6.94–9.48] Mya (node 16) respectively. The Nilo-Sudan region has been recolonized twice in recent times from the Uganda

region (the *N. nubaensis*/*N. ugandensis* clade around 1.13 [0.85–1.53] Mya (node 23) and the *N. rubroreticulatus* complex around 1.62 [1.07–2.22] Mya (node 26). Within the *N. nubaensis*/*N. ugandensis* clade, *N. ugandensis* (Apapi) has dispersed back from the Nilo-Sudan region into the Ugandan region. There were two independent dispersals back into the Coastal region from the Uganda region: *N. usanguensis* at 3.88 [3.09–4.82] Mya (node 18) and *N. angelae* at 1.53 [0.41–2.62] Mya. There was one dispersal back into the Coastal region from the Limpopo region (*N. krysanovi*). The Kalahari region was colonized once at 3.52 [2.96–4.15] Mya (node 30).

3.5. Evaluating the congruence between geotectonic and cladogenetic events: Geobiotic consilience

The reshaping of the East African landscape by the EARS, across a range of spatial scales, includes key landforms formed by more locally confined faulting and/or uplift events. Range-restricted species of *Nothobranchius* are tightly associated with these wetlands and/or rifted features. These are interpreted to represent geobiotic events (Cotterill, 2006; Cotterill and de Wit, 2011). We inferred relationships between key landforms and formative speciation events from cladogenetic evidence (numbered to correspond to the clades, Fig. 4, and taxon localities, Figs. 2A and 2B). Cross-referencing these three figures, to precede detailed discussion below, we describe five principal clusters of geobiotic links in the following paragraphs.

The overlapping ranges of *N. microlepis* and *N. bojiensis* restricted to coastal northern Kenya and Somalia are indicated on the map as 3m/b and the approximated range of *N. virgatus* is indicated as 3v, which diverged in cladogenetic event 3 in response to the tectonic event C, i.e. closure of the drainage of Palaeo-Tana River.

Furthermore, along the Tanzanian coast, the cladogenetic events 13, 15 and 16 link speciation of the three species pairs of *N. insularis* (13i) and *N. lucius* (13l); *N. flammicomantis* (15f) and *N. guentheri* (15g); and *N. korthausae* (16k) and *N. ruudwildekampi* (16r). These cladogenetic events correspond to the isolation of the mainland from Mafia Island (13 and 16) and Zanzibar Island (15). These islands represent the Mafia and Zanzibar horsts that formed in tectonic event M (deepening of the Pangani Offshore Rift), and thereafter subject to the interplay between sea-level changes (eustasy) and recurrent tectonics have been intermittently linked with the mainland (Prendergast et al., 2016).

In southern Tanzania and northern Mozambique, three speciation events are associated with the eastern rim of the Malawi horst. These include: *N. kilomberoensis* (12k) and *N. niassa* (12nia) that correspond to the tectonic event I. Together with *N. wattersi* (8w) and *N. kirki* (8k), south of Lake Malawi (tectonic event J), *Nothobranchius sp.* Northern Mozambique (9nmsp) is confined downstream of the watershed formed by tectonic event H. Focused along the Chembe-Chingale fault (J), skirting the Shire valley, this uplifted the Zomba horst and the Chilwa plateau and also broke and/or reversed flow direction in the Lugenda and Lurio (H) and Ruhuhu (I) rivers.

In central Tanzania, the range of *N. steinforti* (17st), sister to the whole Inland clade, is confined within a landscape that has experienced repeated tectonic instability. The collective geomorphological evidence points to considerable elevation of this plateau, which reversed river flow away from the coast inland into the Uganda region. Northeast, the

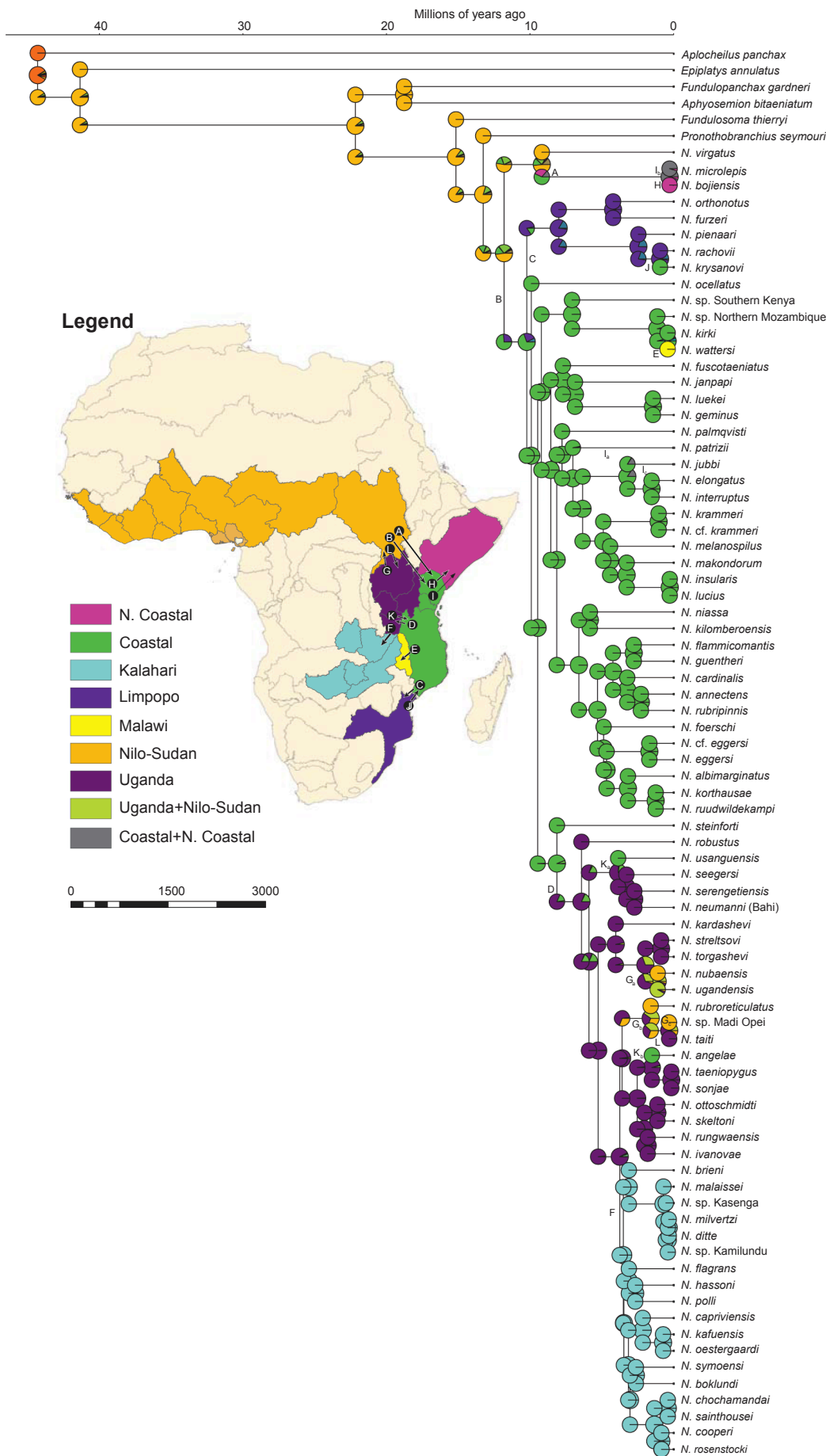


Fig. 5. Ancestral area estimation of *Nothobranchius* reconstructed by BioGeoBEARS under the DEC + j model (AIC = 182; LnL = -88.0232), against a geological time scale (Top). Area coding follows Thieme et al. (2005) and Abell et al. (2008) as indicated in the inserted map including the hypothesized dispersal route derived from the ancestral area estimation. The colour coded ecoregions correspond with the colours of the pies with the exception of the colour coding of India (red) which is not shown on the map. The size of the slices of the pies indicates the relative probability of respective states. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respective *N. neumanni* populations (20nb and 20nm in the Bahi and Manyara graben, respectively) are isolated by the watershed separating the endorheic drainage of Lake Manyara (D) from the coastal drainage of Lake Bahi (E).

A number of informative cladogenic events are centred in wetlands isolated by tectonic events across the rifted landscape inhabited by the Kalahari Clade. South of Lake Tanganyika, the ranges of *N. oestergaardi* (32o) and *N. kafuensis* (32k) are revealed to be widely isolated vicariant lineages in the Kafue basin and Mweru-Wantipa basins (Nd), respectively. Further south, in the Caprivi region of Namibia, *N. capriviensis* (31c) is isolated southwest of the Upper Zambezi in the ancient megafan deposited by the south-westerly flow of the Palaeo-Chambeshi that disgorged into the vast palaeo-lakes of the Makgadikgadi graben. Together, the common ancestry of all three species corresponds to the former continuity of the Palaeo-Chambeshi drainage system, prior to tectonism (Cotterill, 2003a,b, 2005, 2006). To the east, *N. boklundi* (33bk) and *N. symoensi* (33s) are isolated in the Luangwa graben and on the Muchinga Escarpment, respectively. Northwest, the isolated ranges of *N. brieni* (28br) and the complex of *Nothobranchius* species in the Lake Mweru Complex (28LMw, tightly distributed around Lake Mweru) correspond to tectonic events Na and Nb, which isolated Proto-Lake Mweru and reversed a precursor of the Lufira River, to flow northwest into the Upemba (=Kamalondo) graben (Cotterill, 2005, 2006).

Collectively, the above-mentioned patterns (cross-referenced in Figs. 2A, 2B and 4) reveal strong congruence between geological and biological events across a landscape reshaped by Neogene rifting.

4. Discussion

4.1. New primer design

The variability of the COI gene underlies previously problematic amplification of partial gene sequences (Dorn et al., 2014), but our primer design specific to *Nothobranchius* overcame these problems. We designed primers that bind to conserved tRNA coding gene regions upstream and downstream of the COI gene. The complete COI gene could then be amplified almost without fail from all samples as one amplification product and sequenced using further internal primers. These internal primers bound to the COI genes of all *Nothobranchius* species (and some outgroups). This strategy of redesigned taxon-specific primers also succeeded in amplifying mitochondrial ND2 and the nuclear Glyt, MyH6 and SNX33 exons across almost all species in the genus. This strategic approach to primer design and taxon-specific primers for the amplification of both mitochondrial and nuclear genetic markers may find much broader application in future phylogenetic analyses, not only of fishes.

4.2. Phylogenetic analysis: Its implications for taxonomy

The phylogenetic hypothesis of *Nothobranchius*, recovered in this study, shares broad similarities with that of Dorn et al. (2014). The differences are likely explained by our greater taxon sampling and more improved genomic evidence, which also resulted in better supported trees. The Southern Clade *sensu* Dorn et al. (2014) (restricted to Mozambique and South Africa) is represented in this study by only five (of seven) species; because its monophyly and species composition has been studied extensively (Dorn et al., 2011; Bartáková et al., 2015). Our analyses retrieved an additional clade, the Coastal-Inland clade, of which *Nothobranchius kirki* and *N. wattersi* were previously included in the Inland Clade by Dorn et al. (2014). Compared to the Cui et al. (2019) study, we retrieved a basal position for *N. virgatus* as opposed to their terminal position. We found our COI and ND2 sequences of *N. nubaensis*, to be identical to the sequences of their *N. virgatus*. Both species occur in Sudan and Ethiopia. This error was subsequently corrected by Cui et al. (2020) after resequencing. However, the species analysed by Dorn et al. (2014) could not be verified independently, in the absence of respective voucher specimens deposited in curated museum collections (cf. Ruedas

et al., 2000). In fact, many *Nothobranchius* sequences in Genbank originate from unreliable aquarium stocks, not vouchered specimens, which obviates robust taxonomic verification.

Our expanded and more resolved phylogenetic hypothesis for the genus also has implications for the subgeneric classification in the genus *Nothobranchius*. Costa (2018) proposed a revised subgeneric classification based on 138 morphological characters and the phylogeny of Dorn et al. (2014). Our phylogenies (maximum likelihood and Bayesian) support the subgenera *Plesiobranchius* and *Cynobranchius* in the Northern clade, the subgenus *Nothobranchius* representing the Southern Clade, the subgenus *Paranothobranchius* representing the *Nothobranchius ocellatus* species group (Watters et al., 2020) and the subgenus *Zononothobranchius* representing the Inland Clade. However, the greater taxon and consequently greater resolution of our phylogenetic hypothesis does not support the subgeneric delimitation of subgenus *Adiniops* by Costa (2018). His proposal for a subgenus *Adiniops* conforms to the Coastal Clade, invoking support by the molecular data of Dorn et al. (2014). Costa (2018) found morphological support to recognize the more slender surface dwelling members in the subgenus *Aphyobranchius*; yet in the absence of relevant molecular evidence he synonymised *Aphyobranchius* with *Adiniops*.

In contrast, our phylogenetic hypothesis retrieves a monophyletic *Aphyobranchius*, albeit including *Nothobranchius fuscotaeniatus*. However, the morphology, habitat preference and breeding behaviour of this species (e.g. trapezoidal anal fin, pointed snout, preference for a thickly vegetated habitat, mid to upper water spawning tendency (Watters, 2016)), is similar to the other three *Aphyobranchius* species, *N. luekei*, *N. janpapi* and *N. geminus*, but was not assessed by Costa (2018). We argue this synonymisation is premature. Furthermore, Costa (2018) includes *N. wattersi* and *N. kirki* in subgenus *Adiniops*, but our phylogeny retrieves these species together with *Nothobranchius* sp. “Southern Kenya” and *Nothobranchius* sp. “Northern Mozambique” in a separate well supported Coastal-Inland Clade. The morphology of these species will have to be reassessed carefully before any subgeneric delimitation of the genus can be finalized. Whilst the relationships of the Coastal-Inland clade, the clade representing subgenus *Aphyobranchius* and the Coastal Clade that consists of members of subgenus *Adiniops* are resolved in our Bayesian phylogeny (Supplementary Fig. 1), the relationships of these groups are not well supported in the maximum likelihood phylogeny (Fig. 3A). In conclusion, more molecular investigations are required to accurately delimit any subgenera of *Nothobranchius*.

4.3. Origins and biogeography of *Nothobranchius*

Our estimated age of the genus *Nothobranchius* is 13.27 [11.51–15.14] Mya. Helmstetter et al. (2016) dated the age of the genus as slightly older at 14.15 Mya, but their sampling analyzed only one *Nothobranchius* species. Dorn et al. (2014) approximated the age of the genus as 8.32 [5.92–10.75] Mya which is much younger, but this result reflects their selection of outgroups.

Our ancestral area estimation using the DEC + *j* model, constrains the Miocene origin of *Nothobranchius* in the Nilo-Sudan region, contrary to the East African Coastal area suggested by Dorn et al. (2014) using DIVA analysis. Dorn et al. (2014) sampled 22 taxa (including *N. kirki* and *N. wattersi*) in the Coastal Clade, and only 12 species in the Inland Clade. In contrast, our BioGeoBears analysis compared 30 species from the Coastal clade, a further four in the Coastal-Inland Clade (including *N. kirki* and *N. wattersi*), *N. ocellatus* and 37 species in the Inland Clade. Our ancestral area estimation used eight areas in contrast to the ten assigned by Dorn et al. (2014), some of which were subdivisions of the standardized ecoregions of Abell et al. (2008) and Thieme et al. (2005). Furthermore Dorn et al. (2014) did not code the ancestral areas of their outgroups, thereby excluding them from their ancestral area estimation. We conclude all these factors acted in concert to incorrectly derive the East African Coastal region as the ancestral area of *Nothobranchius*.

The ancestral area estimation constraining a Nilo-Sudanian origin of the genus *Nothobranchius* points to its possible origin (together with its immediate ancestors, *Pronothobranchius* and *Fundulosoma*) in the Miocene drainages of mega Palaeo-Lake Chad (K, Fig. 2A; Griffin, 2006; Talbot and Williams, 2009; Partridge, 2010); so *Pronothobranchius* and *Fundulosoma* could have reached their present locations by westward dispersal from the Volta drainage basin in West Africa across the ancient Niger drainage basin; as the topology of Niger, Senegambia and Volta has barely changed since the Early Oligocene, 29–34 Mya (Chardon et al., 2016). The ancestor of genus *Nothobranchius* is likely to have reached the Sudd Basin via the Nilo-Sudan dispersal route, a route also exploited by other fish groups (Schilbe, Van der Bank et al., 2012 and Synodontis, Pinton et al., 2013).

In the Northern Clade, we retrieved a deep branch between *N. virgatus* in the Sudan, and *N. microlepis* and *N. bojiensis* restricted to coastal northern Kenya and Somalia (defined as the Northern Coastal region). The ancestral distribution of this clade corresponds to range expansion via an ancient drainage system across this region (from Sudan and to northern coastal Kenya i.e. the Coastal region). The Palaeo-Tana River was disrupted by the inaugural event that established the first lakes in the Turkana rift in the Early Miocene between 12 and 8 Mya (Tiercelin and Lezzar, 2002; Macgregor, 2015). Volcanism and tectonics finally closed the second outflow from the Turkana basin during the late Pliocene to early Pleistocene (ca. 2 Mya) isolating the Palaeo-Tana River from the Turkana basin (Bruhn et al., 2011).

The founding radiation in the Coastal area at ~12 Mya would also have been from the catchments of the Upper Nile by dispersal along the route via the Palaeo-Tana River across northern Kenya to the coast. Peripatric speciation associated with dispersal along the East African coast founded the Coastal Clade, across the catchments of the Rufiji-Ruaha and Ruvu rivers (M). In the case of the Southern Clade which occurs in the Limpopo region, we postulate southward dispersals, contingent on the Miocene uplift that inaugurated the southern extension of the EARS through the Tanganyika and Malawi graben.

Dispersal to the Limpopo region was possibly via a stepping-stone mode (for which we find no evidence) or a single long-distance dispersal event (by flooding of the landscape during monsoon and hurricane type precipitation as currently occurs every 50–100 years in coastal Mozambique). More recent dispersals within the Southern Clade in the Limpopo region, are attributed to the interplay between isolation versus widespread flooding within and across easterly flowing rivers (Zambezi, Pungwe, Save and Limpopo) (Bartáková et al., 2015). Although these authors interpret the change in nuclear microsatellite frequencies in the three species complexes to be the result of allopatric (i.e. dichopatric) speciation, the nuclear microsatellite frequencies of populations that have dispersed further south are subsets of their northern ancestors. This genetic structuring points instead to the outcome of peripatric speciation, which would concur with our proposals for this mode of speciation in *Nothobranchius* being associated with broad dispersal processes.

There are, however, numerous instances of allopatric speciation, particularly in the Kalahari Clade (two such cases are presented in greater detail below). Landscape warping, for example, subdivided the formerly contiguous tributaries of the Luapula River in northern Zambia to isolate *Nothobranchius cooperi* from its sister species, *Nothobranchius rosenstocki* (Nagy et al., 2017). Similarly, incremental breakup of the Palaeo-Chambeshi isolated the sister species *N. capriviensis*, *N. kafuensis* and *N. oestergaardi* in respective floodplains. The ancestors of these species must have first had wider distributions, but tectonic events led to their isolation and allopatric speciation.

The Coastal-Inland Clade, with species found from Southern Kenya to Inland Malawi appears as a less well-defined geographic pattern compared to the Coastal and Southern Clade. We attribute the Late Miocene divergence of this clade to inaugural uplift focused on the Malawi-Rukwa-Rungwe triple Junction (Goodier et al., 2011; Macgregor, 2015) which would have disrupted formerly southwest flowing tributaries (including rivers formerly flowing into the Luangwa), and

then Proto-Zambezi River (Key et al., 2015). The isolation of the clade comprising the two sister-species *N. wattersi* and *N. kirki*, is most plausibly explained by an older drainage connection linking southern Malawi through the Luito and Lugenda rivers (H) and northern Mozambique. In turn, *N. wattersi* and *N. kirki* were separated 0.44 [0.24–0.68] Mya (node 8) on either side of the upthrown east sloping horst (including the Zomba Plateau) represented in the Chembe-Chingale fault bounding the Shire valley (J, Fig. 2B), which separates the Chilwa basin from drainage associated with Lake Malawi (Bloomfield, 1965). The early Pliocene divergence of *N. sp.* “Southern Kenya” can be explained by the reshaping of the regional drainage extending north from the Malawi rift across northern Mozambique and Tanzania. Uplift of east-bounding horsts of the Malawi rift valley separated and reversed flow in precursors of the Lugenda and Kilombero-Ruhuhu rivers (I, Fig. 2A and 2B; Cotterill, unpublished data). The latter rivers together with neighbouring portions of the Ruaha and Rufiji rivers, survive on the inland plateau as inherited vestiges of these antecedent drainages (Key et al., 2015). *Nothobranchius* sp. “Southern Kenya” most likely represents the surviving representative of ancient populations of *Nothobranchius* formerly more widely distributed in these antecedent wetlands.

This hypothesis is supported by the isolation of *N. steinforti* and *N. kilomberoensis* restricted to seasonal ponds in the Upper Wami and Upper Kilombero rivers, respectively (isolated by Neogene tectonism (Le Gall et al., 2004)); where their ranges are congruent with the relictual endemic cichlid, *Haplochromis vanheusdeni* (Schedel et al., 2014). The deeply divergent clade of *N. kilomberoensis* and *N. niassa*, which diverged at 5.85 [4.54–7.25] Mya (node 12), exhibit a congruent relationship within the Coastal Clade. Moreover, the widespread, monophyletic *Nothobranchius ocellatus* represents a single relictual lineage; the range and age (9.92 [8.54–11.29] Mya (node 10)) of its populations presents further concordant evidence for a wider ancestral distribution of this complex of lineages. Prior to inception of the EARS, the precursor of the Kilombero and neighbouring rivers flowed west within a wider drainage network into the Proto-Zambezi system (Key et al., 2015).

A dispersal to inland areas in present-day Tanzania (corresponding to the current distribution area of *N. steinforti*, 17st, Fig. 2A) at 8.14 [6.94–9.48] Mya (node 17) was likely followed by uplift, which then facilitated new drainage links into the Lake Victoria basin. No other connection between the Coastal and Ugandan Region was present during that timeframe; this dispersal from the area within the Coastal region was also identified by Dorn et al. (2014) and inaugurated three radiations in the Inland clade associated with dispersal across the Uganda region (from the Lake Victoria basin) along the north-flowing drainages of the Upper Nile at 6.43 [5.40–7.61] Mya (node 19). Four subclades occupy senile drainage basins immediately north and south of Lake Victoria (the *N. neumanni*, *N. ugandensis*, *N. rubroreticulatus* and *N. taeniopygus* complexes). One notable dispersal of the Inland Clade likely through the equatorial Nile extended beyond southern Sudan into the Lake Chad drainages in the northwest (K, Fig. 2A) in the Nilo-Sudan region (*N. rubroreticulatus*) at 3.60 [3.01–4.27] Mya (node 25), whilst its sister species, *N. sp.* Madi Opei and *N. taiti* are restricted within the Nile drainage, indicating a north-westerly drainage dispersal of *N. rubroreticulatus*: in the opposite direction to ancestral dispersal of the genus. A second dispersal into Sudan (*N. nubaensis/N. ugandensis*) occurred at 1.13 [0.80–1.53] Mya (node 23). Concurring with findings in both *Hydrocynus* (Goodier et al., 2011) and *Synodontis* (Pinton et al., 2013), this finding in *Nothobranchius* testifies to the relatively high porosity of the Sahelian landscape to widespread movements by fishes.

The dispersal to the Kalahari Plateau in the Pliocene at 3.52 [2.96–4.15] Mya (node 30) was most likely via a southwest flowing tributary of the Palaeo-Chambeshi drainage system from southwest Tanzania across the Mporokoso Plateau (Zambia) to reach southeast Congo headwaters and eastern Namibia (Caprivi) and gave rise to a radiation within the Inland Clade, which straddles the eastern portion of the vast Kalahari Plateau. Extant representatives include *N. oestergaardi*,

N. kafuensis and *N. caprivensis*, whose vicariant distributions are congruent with a formerly widespread occurrence along the primary axis of the endorheic, southwest-flowing Palaeo-Chambeshi River. The speciation of the closely related species complexes, comprising *N. brieni*, *N. chochamandai*, *N. cooperi*, *N. hassoni*, *N. malassei*, *N. polli* and *N. sainthouei*, can be explained by captures and/or reversals of headwaters that formed the south-flowing Trans-Katanga network draining the Katanga and Mporokoso plateaux (southern Katanga and northeast Zambia) then linked with the Palaeo-Chambeshi River. Collectively, these headwaters included precursors of the Upper Chambeshi, Upper Lufira, Upper Luapula, and Proto-Luongo rivers draining via the Upper Kafue into a vast Palaeo-Lake complex (Deception and Makgadikgadi) in present-day Botswana (Cotterill, 2003a; Cotterill, 2004; Cotterill, 2005; Cotterill and de Wit, 2011; Moore et al., 2012, Fig. 2B).

Through the Late Neogene, the Palaeo-Chambeshi network was disrupted by sequential diversions of its headwaters into the Upper Congo and Middle Zambezi rivers. The collective outcome isolated all Trans-Katanga tributaries north of the Kafue Flexure, represented in the Zambezi-Congo watershed (Cotterill, 2003a, 2004, 2005; Cotterill and De Wit, 2011; Moore et al., 2012, Fig. 2B). This radiation includes the sister species *N. symoensi* and *N. boklundi*, which speciated at 2.65 [2.02–3.30] Mya (node 34): isolating *N. boklundi* in the Luangwa graben. The vicariance of *N. boklundi* is most plausibly explained by beheading of eastern headwaters of the Palaeo-Chambeshi system by Late Pliocene uplift of the Muchinga Escarpment (O, Fig. 2A), which deepened the Luangwa rift significantly (Daly et al., 2020).

This mechanism further explicates the biogeographical anomaly, pointed out by Skelton (1994), of a past linkage between the ichthyofaunas of the Lower Zambezi and Upper Congo systems. The historical affinity of these assemblages is represented by several genera of central African fishes, including cichlids, cyprinids, mormyrids and silurids (see Skelton, 1994, 2012) and also freshwater molluscs, Unionidae; notably a clade of Coelaturini, in which the divergences between Malawi-Lower Zambezi and Upper Congo lineages are constrained in the Early Pleistocene (Ortiz-Sepulveda et al., 2020). Subject to further sampling in the Zambian Congo drainage of the characiform fish, *Distichodus fasciolatus* (widespread in the Congo basin), we interpret its Early Pleistocene divergence from the Lower Zambezi endemic, *D. shenga*, as further congruent support of this former biogeographical link (Arroyave et al., 2020). Recently, Daly et al. (2020) integrated independent lines of geophysical and structural evidence to reconstruct the tempo and mode of southwest propagation of the EARS across the Kalahari Plateau through the late Neogene. Their new structural evidence links uplift of the Muchinga Escarpment as integral to the warping of the Kafue Flexure. Initiation of this regional rifting event is constrained as Late Pliocene (3–2.5 Ma), which agrees with the molecular dating of speciation in *N. boklundi* and molluscs.

Indeed, Otero (2010) in her seminal paper on the African fish fossil record, commented how biogeographical signals of freshwater fishes could serve as proxies to reconstruct the history of drainage systems. As explained above, and demonstrated for New Zealand galaxiid fishes (Craw et al., 2008, 2019; Waters et al., 2020) and many endemic biota coevolving within confining habitats along the San Andreas fault system of western North America (Gottsocho, 2016), freshwater fishes exemplify stenotopic organisms. Thus, stenotopy renders freshwater fishes highly sensitive to the dynamic constraints of continental topography: shaped by tectonics, climate and eustasy. In this context, in light of all of the geobiotic evidence, the diversification of *Nothobranchius* recapitulates geologically recent drainage history across the African continent. These fishes can serve as one of the most informative indicators of landscape evolution.

4.4. Geobiotic consilience between geotectonic and cladogenetic events

Our age estimate of *Nothobranchius* - and inclusive speciation events - exhibit tight synchrony with many of the changes to key landforms

across the landscapes of East Africa through the Neogene; especially during the Late Miocene (~7–5 Mya). This was when recurrent rifting, uplift and warping modified the topography of southern and central Africa dramatically (Partridge et al., 1995; Partridge, 1998, 2010; Macgregor, 2015). We suggest that one of the poignant outcomes for *Nothobranchius* that followed on the uplift, was to reposition the drainage systems of inland Tanzania at higher elevations. Ensuing dispersals from these uplifted plateaux would have enabled killifishes to disperse westwards back into the upper Nile drainages (in the Inland Clade, and thereafter southwards and again northwards). Therefore, besides the flooding hypothesis that explains dispersals from a drainage colonized in an ancestral dispersal, we invoke a further hypothesis to explain both vicariance (=dichoptry) and peripatry (*sensu* Cracraft, 1984) across drainage systems. Our tectonic hypothesis argues that recurrent tectonism, causing regional uplift and rifting, repeatedly reconfigured drainage systems across the south-central African plateau. Thus, we consider landscape evolution, operating across a range of spatial scales, to be the primary cause of speciation in *Nothobranchius*.

This causal linkage of tectonics driving speciation of these African fishes concurs with several other species complexes (Cotterill and De Wit, 2011). These include *Mastocembalus* eels (Brown et al., 2010; Day et al., 2017), cichlids (Hermann et al., 2011; Schwarzer et al., 2012), the characin tigerfishes *Hydrocynus* (Goodier et al., 2011), the mochokid silurid genus *Synodontis* (Pinton et al., 2013) and freshwater molluscs, Unionidae (Ortiz-Sepulveda et al., 2020). The diversification of African cichlids reveals complex patterns of allopatric speciation and hybridization not only in rift valley lakes, but also the continent's principal drainage basins. The latter riparian cladogenic events are interpreted as the outcomes of drainage rearrangements; where pulses of hybridization are explained by local panmixis of distinct assemblages between headwaters during river capture events (Schwarzer et al., 2012; Weiss et al., 2015; Schedel et al., 2019; Schedel et al., 2020). If relevant, long-distance dispersal would have presented in different species, with geographically disjunct distributions (localities scattered across widely separated ranges). As this was not found, the results of this study concur with the tectonic and flood dispersal hypotheses and reject the mammal and bird-related dispersal hypotheses. Moreover, our tectonic hypothesis provides a robust explanation for the greater majority of the speciation and cladogenic events, which have shaped the diversity of *Nothobranchius*.

Another interesting finding reveals *Nothobranchius* lineages reached the rifted horst islands of Mafia and Zanzibar from the mainland on at least three separate occasions (from as far back at 2.79 Mya to as recently as 29 kya). The geological evidence for the timing(s) of isolation of these offshore horsts is poorly constrained with respect to the timings of biotic isolations caused by rifting and/or marine highstands. The repeated isolations of these killifishes can be attributed to recurrent pulses of rifting, in concert with sea-level changes, starting ~3.0 Mya (De Menocal, 2004), when glacial lowstands of the Indian Ocean formed land bridges to the mainland. These molecular dates for the offshore endemics of *Nothobranchius* are therefore particularly revealing. They provide the first empirical estimates for when mainland bridges linked these islands, whose biodiversity is characterized by many other endemics (Kingdon, 1990; Prendergast et al., 2016, Fig. 4).

However, climate has also been an important mechanism. South-central Africa has also been exposed to recurring episodes of aridification (Partridge et al., 1995; Partridge, 2010), which is borne out by repeated radiations of arid-adapted biota such as plants (Schrire et al., 2009; Thiv et al., 2011; Bellstedt et al., 2012) which dispersed across the central African 'Arid Corridor' (Verdcourt, 1969; White, 1983). This arid corridor also served as a dispersal route for arid-adapted vertebrates including mammals (Roberts, 1936; De Menocal, 2004; Bobe, 2006), e.g. ground squirrels (Herron et al., 2005), spring hares (Matthee and Robinson, 1997) and gemsbok (Iyengar et al., 2006). However, in place of gradual aridification, cryosphere expansions caused episodes of greater aridity that interleaved with more mesic episodes during the Plio-

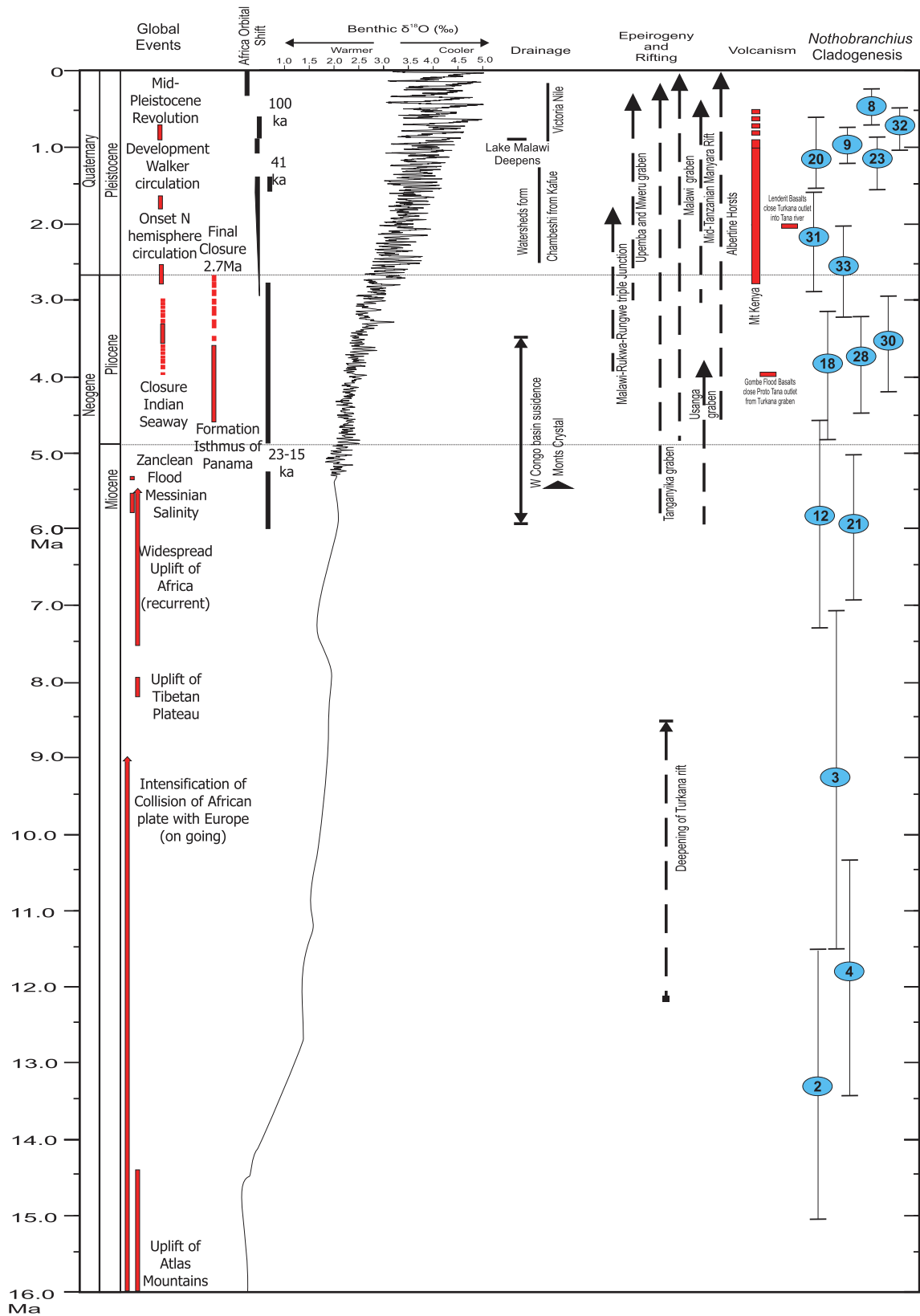


Fig. 6. Timeline of selected palaeoenvironmental events and trends since the Early Miocene compared against selected cladogenic events of *Nothobranchius* (estimated dated nodes correspond to Fig. 4). The former include major tectonic events, notably in the East African Rift System, palaeoclimate and orbital cycling. These profiles highlight the overall cooling trend in global climate in relation to selected episodes of epeirogeny, rifting and volcanism. Sea surface temperature (Benthic $\delta^{18}O$) is plotted from Lisiecki and Raymo (2005). See Fig. 2 for details and references, with addition of Kingston (2007), Golonka (2009), Gaina et al. (2013), and Joordens et al. (2019).

Pleistocene (De Menocal, 2004; Lyons et al., 2015; Joordens et al., 2019).

Nevertheless, the establishment of widespread savannah conditions in the mid-Neogene set up the persisting conditions that have fostered the radiation of *Nothobranchius* across tropical east and central Africa. Seasonal aridification is invoked as the ultimate cause of the inaugural evolution of annualism in both the African Nothobranchidae, and neotropical Rivuliniidae (characterized by diapause as a key stage in the life history development: Peters, 1963; Watters, 2009; Furness et al., 2015). Helmstetter et al. (2016) showed that adaptations for annualism evolved repeatedly in distantly related ancestral cyprinodontiforms during the Palaeogene; besides three times in the neotropical Rivuliniidae, at least twice in the African Nothobranchidae (once in the ancestor of genus *Nothobranchius* and its sister genus *Pronothobranchius*) and, independently, twice, in the West African genus *Fundulosoma*. Annualism is therefore not a plesiomorphic mechanism inherited by all Nothobranchidae, which enables populations to persist in seasonal tropical wetlands. It follows that this adaptation cannot be invoked as the primary agent of speciation in *Nothobranchius* (contra Dorn et al., 2014). Instead, our combined geobiological evidence identifies the interplay between episodic dispersals and vicariance driven by tectonics to be the primary causes of speciation.

We further underscore the vulnerabilities of killifishes to the repeated catastrophic eruptions of no less than 615 volcanoes (see Scoon, 2018), which have impacted at regional scales across the landscape of central Africa: particularly along the arms of the Albertine, Ethiopian and Gregory Rift systems (Tiercelin and Lezzar, 2002; Maslin, et al., 2014; Macgregor, 2015). The precise geochronological dates of volcanic eruptions across the North Tanzanian Divergence Zone (Taylor et al., 2014; Mana et al., 2015) coincide with episodes of regional uplifts during the Plio-Pleistocene. Repeatedly, the sub-Plinian and Plinian eruptions that have dominated the intense volcanism of the Gregory Rift smothered vast areas under alkaline pyroclastics (Scoon, 2018). They coincided with faulting and warping of the land surface, which acted to drain and/or impound major endorheic Mega-Lakes within the North Tanzanian Divergence Zone: notably Manonga (E, Fig. 2A and 2B) and the precursor of Lake Victoria (Johnson et al., 1996; Harrison et al., 1996). Volcanism likely caused extinctions of lineages of *Nothobranchius* in these areas; obviously their magnitude is difficult to assess, but recurring volcanism would have impacted significantly on the present-day species richness of the region. The historical record (~200 years) testifies to the impacts of alkaline volcanism on the biodiversity of northwest Tanzania. These events have been relatively frequent, and locally catastrophic. For example, over five months of 1917, eruptions of highly alkaline soda ash from Oldoinyo Lengai destroyed large areas of grassland and rendered surface water undrinkable for cattle (Peters et al., 2008) and it would have been catastrophic for any *Nothobranchius* killifishes restricted to shallow wetlands of this region.

If the tectonism that reconfigured African drainage basins had had only marginal impacts on ancestral populations of *Nothobranchius*, then we would expect the pattern and process in rifting history to be decoupled from the tempo and mode of the speciation of these fishes across tropical Africa. In contrast, geographically constrained ranges of *Nothobranchius* preserve fingerprints of paleogeographic history of the African Neogene, in which the collective genomic record of extant species testifies to impacts of tectonism across tropical Africa. The phylogeny integrates several lines of consilient evidence for well constrained rifting events, which are inferred to account for cladogenic events. These results reveal the high fidelity - thus consilience - between local geological events with the molecular dates of appearances of respective lineages (and clades). Collectively, with reference to Figs. 2 and 6, the following findings underwrite support of our Tectonic Hypothesis presented above:

- Establishment of the Palaeo-Tana River in the Early Miocene that followed initial rifting of the Turkana graben 12–8 Mya (Tiercelin and Lezzar, 2002; Bruhn et al., 2011) coincided with the estimated

divergence between *N. virgatus* (Sudan) from *N. microlepis* and *N. bojiensis* restricted in northern Kenya and Somalia (C) in the Northern Clade (9.19 [7.05–11.38] Mya);

- The initial uplift forming the Albertine Rift, was coincident with Virunga-Kiwu volcanism (6–5.5 Mya, Macgregor, 2015) and congruent with the first order cladogenesis of the Inland Clade (Early Pliocene, 5.91 [5.00–6.91] Mya);
- Speciation events in the *N. neumanni*/*N. usanguensis* clade are cotermporal with the Plio-Pleistocene faulting of the Mid-Tanzanian Rift. The first divergence at 3.88 [3.09–4.82] Mya (F) was coeval with formation of the Usangu Rift (Delvaux and Hanon, 2003; Le Gall et al., 2004); and the Pleistocene deepening of the Manyara Rift System (Macheyeki et al., 2008) is reflected in divergence between the two lineages of *N. neumanni* in Bahi and Manyara (D, E), respectively (1.07 [0.67–1.53] Mya);
- Divergence of *N. boklundi* from its congeners (2.65 [2.02–3.30] Mya) isolated in the Luangwa graben (33bk), is coeval with speciation events in freshwater molluscs (Ortiz-Sepulveda et al., 2020), which are supported by independent geological evidence (Daly et al., 2020);
- Isolation of *N. sp.* “Northern Mozambique” corresponds to the deepening of Lake Malawi at 800 kya (Ivory et al., 2016), and this rifting likely severed the original headwaters of the Lugenda River (H); it was followed by vicariance of *N. kirki* and *N. wattersi* in the Late Quaternary (0.44 [0.25–0.68] Mya) that coincided with isolation of Lake Chilwa (Watters, 1991) by the watershed in the graben floor (J);
- The timing of separation of the Lake Victoria drainage basin from the White Nile (Talbot and Williams, 2009) is congruent with the molecular divergence of *N. nubaensis* (Upper Nile drainage, A) and *N. ugandensis* (Lake Victoria basin) at 1.13 [0.80–1.53] Mya.

4.5. Conclusions

Our narrative of the evolutionary history of *Nothobranchius* presents the most comprehensive phylogenetic hypothesis of the genus to date. Resolving the causality of phylogenetic events within a chronobiogeographical paradigm (Hunn and Upchurch, 2001; Crisp et al., 2011; Goodier et al., 2011) across an independently constrained time tree demonstrates a robust consilience, in the tight spatio-temporal congruence between rifting and biotic events. These results reveal how recurring tectonism across tropical Africa has been the primary cause of range expansion and speciation in the annual killifish genus *Nothobranchius*: exhibiting tight congruence from the Tana-Turkana region south to the branches of the Okavango and Urema rifts. These analyses clarify species relationships of the major clades in terms of their respective diversification events. This phylogeny identifies that a number of populations are in fact new species. Beyond the scope of the present paper, our phylogenetic work with extensive taxon sampling has resulted in further interesting taxonomic insights (including Nagy et al., 2020 and Watters et al., 2020), which we are currently investigating. A forthcoming paper examines the finer-scaled relationships between tectonism, landscape evolution and *Nothobranchius* diversification in more detail.

Author statement

FPDC and DB conceptualized and designed the study. BN, BW and DB collected fish material in Africa and provided field data. TG supplied fish samples from the Killidata Tissue Sample Bank. PDWvdM performed laboratory analyses under the guidance of DB and FPDC, who also carried out genotyping of many taxa. PDWvdM and FPDC performed phylogenetic analyses. BioGeoBears analyses were conducted by MK and PDWvdM. TF, DS and FPDC performed mapping. FPDC, with BW, DB and TF, reviewed the geological evidence and identified geobiotic linkages. All authors contributed to writing the manuscript and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Specimens for this study were collected in the wild in the African countries in which these fishes occur. The study did not include any species protected by CITES. Most specimens were obtained prior to the legal implementation of the Nagoya Protocol (12.10.2014) and its corresponding EU Access and Benefit Sharing (ABS) regulations, which came into effect on 9th November 2015. Permissions for specimen collection in the wild and exportation of samples were granted for the field trips (A): In 2013, to the Democratic Republic of Congo, with permit number 003/706/INS/AGRI.PEL/KAT.2013; (B). In 2016, to the Democratic Republic of Congo, with research permit number FAC/AGRO/37/2016 and exportation and health certificate number 704/22/SQAV/CP/HKAT/2016; (C) in 2019, to Uganda, Tanzania and Kenya with research permit number CONT/NIC/CMY/1569634 and with health certificate number 2019/CC/OPS/D3/00006 issued to the Royal Museum for Central Africa, Tervuren, Belgium.

Data accessibility

The gene sequences are available in the GenBank repository under the accession numbers as indicated in Supplementary Table 1. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jympev.2020.106988>.

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