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ADVANCES IN CICHLID RESEARCH III

Review Paper

Gondwanan vicariance or trans-Atlantic dispersal of cichlid fishes: a review of the molecular evidence

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Abstract Cichlid fishes are one of the most important model systems for evolutionary biology. Unfortunately, however, the timeline of cichlid diversification is still insufficiently known and limits our understanding of the mechanisms that generated their spectacular diversity. The uncertainty regarding this timeline stems from a decades-old controversy surrounding the phylogeographic history of cichlid fishes. Did cichlid subfamilies diversify as the result of Gondwanan vicariance, as supported by their distribution on former Gondwanan landmasses? Or did they diverge much more recently through oceanic dispersal, as suggested by the fossil record? While a large number of studies have already addressed this question with molecular-clock analyses, no single conclusion has emerged from these investigations. Here, I review the molecular evidence for Gondwanan vicariance or trans-Atlantic dispersal resulting from these studies. I discuss the weaknesses and strengths of each study, aiming to promote the formation of consensus on the matter and to prevent the repetition of previously made mistakes. I find that after accounting for inappropriate calibration strategies and saturation in mitochondrial datasets, the molecular evidence points to trans-Atlantic dispersal long after continental separation, probably around 75–60 Ma.

Keywords Cichlidae · Biogeography · Dispersal · Vicariance · Phylogeny · Bayesian inference · Divergence-time estimation · Fossils

Introduction

With their spectacular species diversity, cichlid fishes are a prime model system for evolutionary research (Schluter, 2000; Seehausen, 2006; Berner and Salzburger, 2015). Over several decades, the field of cichlid research has amassed morphological, behavioral, and genetic data that allowed to reconstruct the evolutionary history of cichlid fishes in fine detail, comparable only to few other particularly well-investigated groups of animals such as Darwin's finches (Grant and Grant, 2008) or great apes (Tuttle, 2014). Cichlid research has thus contributed greatly to our understanding of the roles of ecological opportunity (Wagner et al., 2012), trophic morphology (Muschick et al., 2012), sexual selection (Theis et al., 2017), habitat structure (Sefc et al., 2017), genome architecture (Brawand et al., 2014), and gene flow (Loh et al., 2013; Malinsky et al., 2017; Meier et al., 2017) for the generation of biodiversity. However, while many aspects of cichlid evolution are now comparatively

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well known, a central piece to the puzzle is still missing: a robust and generally accepted timeline of their diversification. This is best illustrated by two studies that both came out in 2013 and estimated the age of the family Cichlidae to be either over 160 million years old (Ma) (López-Fernández et al., 2013) or as young as 45 Ma (Near et al., 2013). This lack of consensus among cichlid researchers is limiting the insights that even recent genomewide datasets can provide. For example, while lake-level fluctuations have often been assumed to drive speciation in the East African Lakes Tanganyika and Malawi (Rüber et al., 1998; Sturmbauer et al., 2001; Ivory et al., 2016; Janzen and Etienne, 2016), all conclusions regarding their role directly depend on the assumed correlation between the times of lake-level changes and speciation events. Thus, without precise estimates for the times of these speciation events the role of lake-level fluctuations cannot be assessed. Similarly, whether lacustrine species radiations occurred in situ or are the result of multiple independent colonization events can only be answered by comparing the ages of the lakes with those of the respective radiations, and wrong conclusions might be drawn if radiation ages are overestimated.

At its core, the lack of consensus regarding the timeline of cichlid diversification stems from a longstanding controversy about the phylogeographic history of cichlids. Because the four cichlid subfamilies Etroplinae, Ptychochrominae, Pseudocrenilabrinae, and Cichlinae (Sparks and Smith, 2004) occur each on a different landmass of the former supercontinent Gondwana (albeit not always exclusively; Fig. 1), it has traditionally been assumed that their divergences were the result of Gondwanan vicariance (Stiassny, 1987; Stiassny, 1991; Zardoya et al., 1996; Streelman et al., 1998; Farias et al., 1999; Farias et al., 2000): that they diverged allopatrically due to the separation of the landmasses inhabited by them. This would imply that the Malagasy subfamily Ptychochrominae diverged from Pseudocrenilabrinae and Cichlinae when Madagascar broke away from Africa, that Pseudocrenilabrinae and Cichlinae diverged when Africa and South America separated, and that within the subfamily Etroplinae the Indian genus *Etroplus* split from the Malagasy genus *Paretroplus* when India and Madagascar broke apart. The timing of these continental separations is comparatively well known: Indo-Madagascar separated from Africa before 150 Ma and reached its current position relative to Africa around 120 Ma (Rabinowitz and Woods, 2006; Ali and Aitchison, 2008; Matthews et al., 2016). Land connections that might have led from Indo-Madagascar to Australia and South America via Antarctica can be excluded after around 100 Ma (Ali and Krause, 2011). The separation of South America and Africa, leading to a continuous North/South Atlantic Ocean, was complete by 105–100 Ma (Moulin et al., 2010; Seton et al., 2012; Heine et al., 2013; Matthews et al., 2016; Olyphant et al., 2017). According to the reconstructions of Heine et al. (2013), this final separation occurred at the location of the Côte d'Ivoire/Ghana Ridge, a protrusion that had resulted from local uplift due to the westward movement of South America. While the now-submerged Rio Grande Rise and Walvis Ridge may have provided stepping stones for dispersal between Africa and South America until around 40 Ma, no continuous land bridge ever existed after the break-up of the two continents (de Oliveira et al., 2009). Finally, India separated from Madagascar 90–85 Ma and the existence of land bridges postdating this split can be excluded (Storey, 1995; Ali and Aitchison, 2008). Thus, according to the Gondwanan vicariance scenario, Ptychochrominae diverged before 120 Ma, Pseudocrenilabrinae and Cichlinae diverged around 105–100 Ma, and the genera *Etroplus* and *Paretroplus* diverged around 90–85 Ma.

However, as pointed out by Murray (2001a), such ancient divergence times for cichlids are not supported by the fossil record. The oldest cichlid fossils are those of five *Mahengechromis* species from the site of the Mahenge paleolake in north-central Tanzania (Murray, 2000a; Murray, 2001b). The age of the lake is well constrained by U-Pb isotopic analysis which dates the lake formation to 45.83 ± 0.17 Ma (Harrison et al., 2001). Conservative estimates of sedimentation rates indicate that the lake would have filled within less than a million years; thus, the age of the fossils most likely dates to 46–45 Ma (Harrison et al., 2001). At least 150 fossil specimens of *Mahengechromis* are known from the locality, allowing a detailed investigation of their morphology (Murray, 2000a). Based on several characters, including the structure of the lower pharyngeal jaw, *Mahengechromis* can clearly be placed within the family Cichlidae; however, its affinities within the family are less certain (Murray, 2001b). Its possession of a single pre-dorsal bone indicates a position among

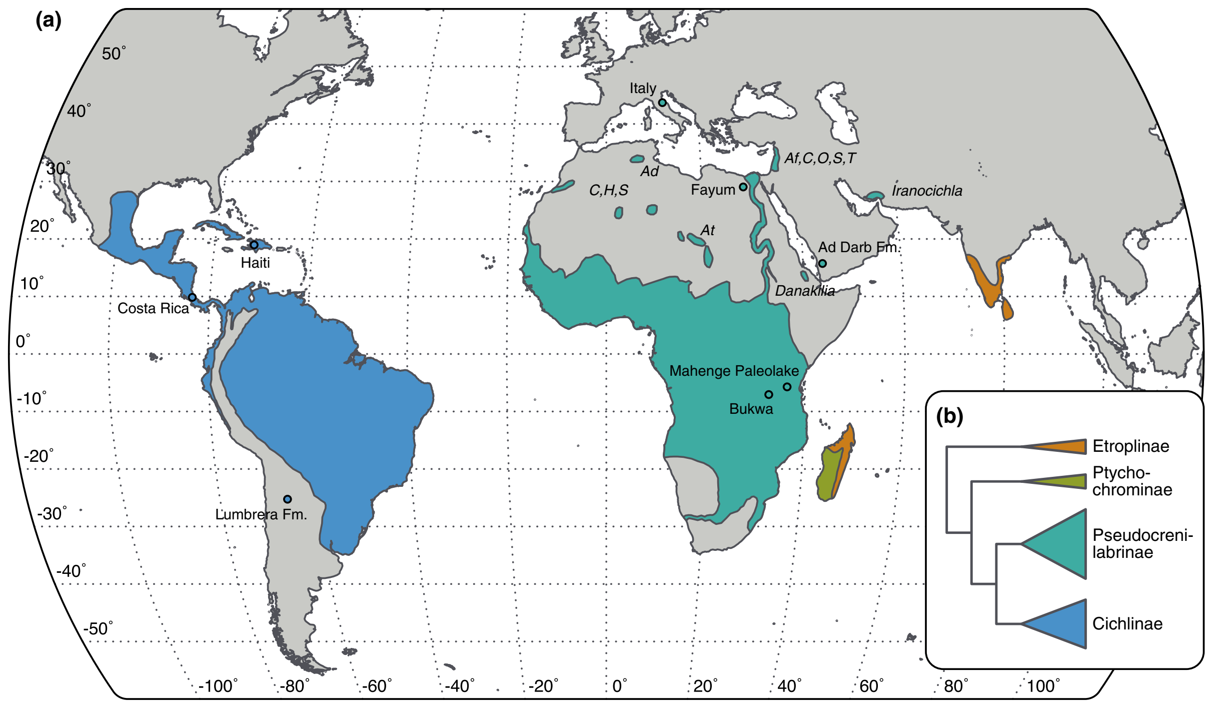

Fig. 1 The global diversity of the four cichlid subfamilies Etroplinae (orange), Ptychochrominae (green), Pseudocrenilabrinae (cyan), and Cichlinae (blue) (Sparks and Smith, 2004). **a** Distribution of the four cichlid subfamilies. Both subfamilies Etroplinae and Ptychochrominae co-occur in the North and East of Madagascar. Introduced populations are excluded. Genus names and abbreviations in italics indicate occurrences at isolated locations: *Astatotilapia tchadensis* Trape, 2016, (*At*) occurs at the Ounianga Serir lakes in northern Tchad (Trape, 2016), and *A. desfontainii* (Lacepède, 1802) is found in Tozeur, Tunisia (Genner and Haesler, 2010). These and other isolated locations in Northern Africa are also inhabited by members of the genera *Coptodon* (*C*), *Hemichromis* (*H*), and *Sarotherodon* (*S*) (Kraiem, 1983; Lévêque, 1990; Dilyte, 2014). In the Levant, *A. flaviijosephi* (Lortet, 1883) (*Af*) occurs together with *Coptodon*, *Oreochromis* (*O*), *Sarotherodon*, and *Tristramella* (*T*) (Goren and Ortal, 1999). The most important fossil locations with cichlid records are indicated with black circles (see Murray, 2001a, for a more complete list), including the Mahenge paleolake and the Lumbrera Formation, from which the earliest records of Pseudocrenilabrinae and Cichlinae, respectively, were reported (Murray, 2001b; Malabarba et al., 2006; Malabarba et al., 2010; Perez et al., 2010). **b** Interrelationships of the four cichlid subfamilies, supported by all recent phylogenetic studies (e.g., Smith et al., 2008; Friedman et al., 2013; Matschiner et al., 2017). Triangle sizes indicate species numbers but are not drawn to scale: For both Etroplinae and Ptychochrominae, 16 species are described, around 2 000 species are known for Pseudocrenilabrinae, and Cichlinae include around 540 species

Pseudocrenilabrinae excluding *Heterochromis* and *Tylochromis*, and analysis of 37 osteological characters provides support for a sister-group relationship between *Mahengechromis* and Hemichromini (Murray, 2000b; Murray, 2001b). This relationship, however, is only slightly more parsimonious than alternative placements and should therefore be considered tentative [(Murray, 2000b), priv.comm.].

Slightly younger than *Mahengechromis* are the oldest South American fossils of cichlids from the Lumbrera Formation in north-western Argentina. Specimens of *Proterocara argentina* Malabarba et al., 2006, *Plesioheros chauliodus* Perez et al., 2010, and *Gymnogeophagus eocenicus* Malabarba et al., 2010 were recovered from the Lumbrera Formation's "Faja Verde" level, a layer of lacustrine origin. The age of this layer has often been associated with the Ypresian–Lutetian boundary (47.8 Ma) (Malabarba et al., 2010; Perez et al., 2010; López-Fernández et al., 2013; Malabarba et al., 2014), but as pointed out by Friedman et al. (2013), Benton et al. (2015), and Matschiner et al. (2017), evidence for this association is lacking. Instead, most authors agree that the Lumbrera Formation should be assigned to the "Casamayoran" South American Land Mammal Age (SALMA) (del Papa et al., 2010, and references there in) which is defined by polarities C20–C18 (Vucetich et al., 2007) and can thus be interpreted as 45–38.5 Ma (del Papa et al., 2010; Bellosi and Krause, 2014). The age of the "Faja Verde" level can be further constrained by absolute U/Pb zircon dating of a tuff layer that lies about 240 m above the fossiliferous level and was measured to be 39.9 ± 0.4 Ma (del Papa et al., 2010). Due to its position relative to the tuff layer, it is clear that the "Faja Verde" level must be older than 40 Ma; however, since no estimates of sedimentation rates are available for the Lumbrera Formation, it is unclear how much older it is. Thus, the age of the cichlid fossils from the Lumbrera Formation can only be constrained to 45–40 Ma. The phylogenetic positions of the three cichlid species from the Lumbrera Formation are relatively well known, owing to the availability of the morphological character matrices for Neotropical cichlids by Kullander (1998) and López-Fernández et al. (2005). Phylogenetic analysis indicated a placement of *Proterocara argentina* within the tribe Geophagini and possibly as the sister group of a clade combining *Teleocichla* and *Crenicichla* (Smith et al., 2008), *Plesioheros chauliodus* was placed within the tribe Heroini, supported by two morphological characters including one synapomorphy (Perez et al., 2010; McMahan et al., 2013), and *Gymnogeophagus eocenicus* was recovered within the genus *Gymnogeophagus* based on five character changes (Malabarba et al., 2010). The latter placement is remarkable as it would indicate that that the extant genus *Gymnogeophagus* existed for over 40 Ma. However, as pointed out by Friedman et al. (2013), erroneous phylogenetic placement due to convergent character evolution, a common observation in cichlids (Muschick et al., 2012), cannot be ruled out.

In addition to the fossils from the Mahenge paleolake and the Lumbrera Formation, many younger remains of cichlids are known, such as a putative *Tylochromis* from the Early Oligocene Egyptian Fayum Formation (Murray, 2002), a putative *Heterochromis* from the Oligocene Saudi Arabian Ad Darb Formation (Lippitsch and Micklich, 1998), *Macfadyena dabanensis* Van Couvering, 1982, from the Daban Series of Somalia that is probably Oligocene in age (Van Couvering, 1982; Murray, 2001a), a putative *Pelmatochromis* from the Early Miocene "Lamitina Beds" east of Bukwa, Uganda (Van Couvering, 1982), *Oreochromis lorenzoi* Carnevale et al., 2003, from the Late Miocene Gessoso-Solfifera Formation of northern Italy (Carnevale et al., 2003), and *Nandopsis woodringi* Gill, 1862, from the Late Miocene deposits at Las Cahobas, Haiti (Cockerell, 1923; Chakrabarty, 2007) (Fig. 1). In total, fossils of at least around 20–30 cichlid species are known (Murray, 2001a). Given that many other percomorph families with great species diversity have no skeletal fossil record at all (e.g., Rivulidae, Atherinopsidae, Gobiesocoidei, Pseudochromidae, Pomacanthidae; see Supplementary Text S2 of Matschiner et al., 2017), the cichlid fossil record is actually relatively rich, despite common claims to the contrary (Koblmüller et al., 2008; Malabarba and Malabarba, 2008; Perez et al., 2010). This means that the assumption of Gondwanan vicariance also requires the postulation of initially long "ghost lineages," which would pose a remarkable contrast to the comparatively rich fossil record after 46 Ma: If Pseudocrenilabrinae and Cichlinae diverged 105–100 Ma with the separation of Africa and South America, both lineages must have existed for around 60 Myr without leaving any fossils. While the probability of this scenario depends on the number of fossil locations with ages between 105–46 Ma that could potentially have yielded fossils of freshwater fishes (Friedman et al., 2013), it is understandable that it is often assumed to be extremely low (Lundberg, 1993; Murray, 2001a). Thus, one out of two apparently highly improbable scenarios is required to explain the phylogeographic history of cichlids: Either (i) cichlids must have traversed the Atlantic Ocean (and probably also the Mozambique Channel and the Indian Ocean) or (ii) cichlids must have occupied both Africa and South America for many tens of million years without fossilization. And it is exactly this paradox situation, where one out of two unlikely scenarios must be true, which has fueled the controversy surrounding cichlid phylogeography for the last three decades (Stiassny, 1987; Murray, 2001a; Chakrabarty, 2004; Sparks and Smith, 2005). Neither advocates of Gondwanan vicariance nor proponents of trans-Atlantic dispersal were willing to accept that the perceived improbable alternative scenario might possibly be true, and at first, little external evidence was added to resolve the controversy. Fortunately, however, recent developments in molecular divergence-time estimation, coupled with the ever-increasing availability of sequence data, have begun to open a way out of this impasse, as I will describe in the next section.

Molecular estimates of divergence times in cichlids

Based on the concept of the molecular clock (Zuckerkandl and Pauling, 1962), phylogenetic divergence-time estimation with cichlid and non-cichlid sequence data allows to place the timeline of cichlid diversification within the larger context of teleost evolution. When this context is adequately established through the use of fossils as calibration points, inferences can be made for divergence times both outside and within Cichlidae. Moreover, if divergence-time estimation is conducted in a Bayesian manner, probabilistic confidence intervals can be estimated and potentially allow to reject certain prior assumptions about divergence times, which therefore enables tests of the Gondwanan vicariance hypothesis. Several such studies have already been conducted with sequence data of African and Neotropical cichlids and I will discuss the results of these below to provide a summary of the molecular evidence for or against Gondwanan vicariance. Because a detailed description of the concepts of Bayesian molecular-clock analyses would be beyond the scope of this review, I will keep the discussion of these technical aspects to a minimum but refer the interested reader to the excellent and comprehensive reviews on the topic by Ho and Duchêne (2014) and Bromham et al. (2018).

I limit this summary to the divergence time of Pseudocrenilabrinae and Cichlinae in relation to the continental breakup of Africa and South America rather than comparing the divergence times of all cichlid subfamilies with the respective continental separations. I do so for three reasons: First, the fossil records of Pseudocrenilabrinae and Cichlinae are relatively well characterized and provide robust minimum ages for the origins of the two subfamilies, but no fossils are known of Ptychochrominae and Etroplinae (Murray, 2001a). Second, less sequence data are available for Ptychochrominae and Etroplinae and few previous studies have aimed to estimate their divergence times. Third, throughout most of the period between 100–50 Ma, the distance between Africa and South America was larger or at least comparable to the distances between Africa and Madagascar or Madagascar and India (Seton et al., 2012). I therefore assume that any conclusion drawn regarding the dispersal or vicariance of African and Neotropical cichlids will likely also be applicable to the divergences of Malagasy and Indian cichlids (however, I acknowledge that a more detailed comparison of the divergence times of Malagasy and Indian cichlids, the distances between continents, and the paleocurrents prevailing at the time may be required to verify this assumption).

Since the publication of the first study on the divergence of cichlids from different continents (Kumazawa et al., 2000), molecular divergence-time estimation has seen tremendous progress, driven by increased availability of sequence data, published surveys of suitable fossil calibrations (Benton and Donoghue, 2007; Benton et al., 2015), and methodological improvements. Major methodological milestones include the development of Bayesian inference tools for divergence-time estimation (first introduced by Thorne et al., 1998, but not applied to cichlid data until 2007), relaxed-clock models (Drummond et al., 2006), and new approaches to integrate fossils into the analysis (Ronquist et al., 2012a; Heath et al., 2014; Gavryushkina et al., 2017; Matschiner et al., 2017). Thus, published studies must be seen in their temporal context, taking into account the sequence data and inference methods available at the time.

The focus of this summary is on a set of eleven studies using Bayesian inference, the results of which are compared in Fig. 2 and Table 1, but two earlier investigations based on rate comparisons are also described to provide context (Kumazawa et al., 2000; Vences et al., 2001). Studies that merely use assumed Gondwanan vicariance for calibration without testing the hypothesis are excluded (Setiamarga et al., 2009; Miya et al., 2010; López-Fernández et al., 2013).

Kumazawa et al. (2000)

To the best of my knowledge, the first study aiming to estimate divergence times of cichlid fishes was published by Kumazawa et al. (2000), as a chapter in the book “The Biology of Biodiversity” (Kato, 2000). Those authors used sequences of the mitochondrial NADH dehydrogenase subunit 2 (ND2) and cytochrome b (cytb) genes (2,049 bp) of 22 species (including 3 Pseudocrenilabrinae and 3 Cichlinae) to estimate a neighbor-joining tree (Saitou and Nei, 1987) and to calculate pairwise genetic distances. Few nodes of the resulting phylogeny received strong support (Bootstrap support, BS > 90), but those six that did all agree with the latest classification of bony

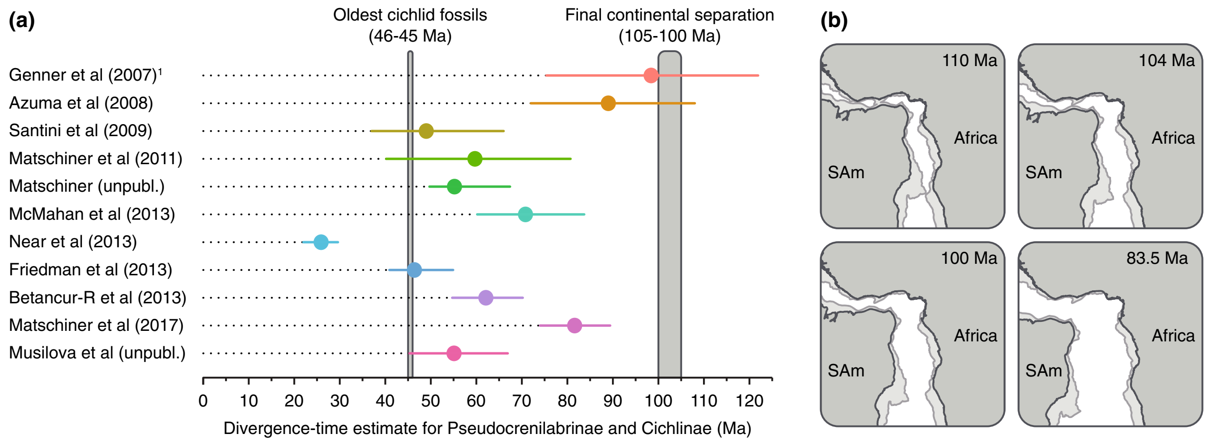

Fig. 2 The divergence of Pseudocrenilabrinae and Cichlinae in relation to continental separation. **a** Molecular estimates for the divergence time of African and Neotropical cichlids from eleven different Bayesian dating studies. Bars indicate 95% highest-posterior-density intervals and dots on these bars represent the mean age estimates (see Table 1 for more details). Gray shapes show the age of the oldest fossil record of cichlids, *Mahengechromis*, at 46–45 Ma, as well as the timing of the final separation of Africa and South America between 105–100 Ma (see Introduction; Matschiner et al., 2017). **b** The process of continental separation of Africa and South America (SAm) according to Heine et al. (2013). Dark gray outlines indicate present-day coastlines; light gray outlines show the landward limit of the oceanic crust. [1]Only the divergence-time estimate based on the Benton and Donoghue (2007) set of calibrations is shown for Genner et al. (2007)

Table 1 Studies reporting Bayesian molecular divergence-time estimates of African and Neotropical cichlid fishes

Study	# taxa	# sites	% complete	# cal.	Software	Clock	Age estimate (Ma)
Genner et al. (2007)[1]	34 (18, 6)	905 (m)	96.0	4	multidivtime	AUTO	98.4 (121.9-75.4)
Azuma et al. (2008)	54 (4, 2)	10 034 (m)	99.9	18	multidivtime	AUTO	89.0 (108.0-72.0)
Santini et al. (2009)	227 (6, 1)	1 445 (n)	93.1	45	BEAST 1.4.6	UCLN	49.0 (66.0-37.0)
Matschiner et al. (2011)	67 (4, 2)	4 599 (b)	93.5	6	BEAST 1.5.3	UCLN	59.7 (80.7-40.2)
Matschiner (unpubl.)	9 (3, 1)	28 239 (n)	92.6	3	BEAST 1.6.1	UCLN	55.2 (67.4-49.7)
McMahan et al. (2013)	133 (19, 57)	2 069 (b)	88.2	4	BEAST 1.6.2	UCLN	70.8 (83.7-60.2)
Near et al. (2013)	579 (2, 3)	8 577 (n)	84.5	37	BEAST 1.6.1	UCLN	25.9 (29.6-22.0)
Friedman et al. (2013)	158 (54, 29)	7 887 (n)	93.7	10	BEAST 1.6.1	RLC	46.4 (54.9-40.9)
Betancur-R et al. (2013)	202 (3, 4)	17 812 (b)	51.5	59	BEAST 1.7	UCLN	62.1 (70.2-54.7)
Matschiner et al. (2017)	366 (64, 79)	27 650 (b)	40.7	147	BEAST 2.3.1	UCLN	81.6 (89.4-74.0)
Musilova et al. (unpubl.)	101 (5, 1)	71 902 (n)	93.6	28	BEAST 2.2.0	UCLN	55.1 (66.9-45.3)

Studies without calibration points outside of Cichlidae or studies using assumed Gondwanan vicariance for calibration (e.g., Schwarzer et al., 2009; Setiamarga et al., 2009; Miya et al., 2010; López-Fernández et al., 2013) are excluded. Taxon numbers in parentheses indicate sampled species of Pseudocrenilabrinae and Cichlinae. Labels "m," "n," and "b" following site numbers indicate that datasets include mitochondrial (m) or nuclear (n) sites, or both (b). *AUTO* autocorrelated clock model; *UCLN* uncorrelated lognormal clock model; *RLC* random local clock model. [1]Only the age estimate based on the Benton and Donoghue (2007) set of calibrations is given for Genner et al. (2007)

fishes (Betancur-R et al., 2017). Distances of amino-acid sequences were compared with assumed ages at three nodes: the divergence of bony fishes (Osteichthyes) and sharks (Chondrichthyes) with an assumed age of 528 Ma; the divergence of ray-finned fishes (Actinopterygii) and lobe-finned fishes (Sarcopterygii) with an assumed age of 450 Ma; and the divergence of Pseudocrenilabrinae and Cichlinae with an age according to the Gondwanan vicariance hypothesis of around 100 Ma. The ages of the first two nodes were taken from Kumar and Hedges (1998), a study that was later heavily criticized for its use of a single-age calibration without accounting for its uncertainties (Graur and Martin, 2004). The comparison of Kumazawa et al. (2000) indicated that substitution rates inferred for the three nodes were similar, which was taken as support for the assumed divergence time of Pseudocrenilabrinae and Cichlinae, and thus for the Gondwanan vicariance hypothesis.

While the study by Kumazawa et al. (2000) was a remarkable step forward for its time, its results may be biased due to the few and questionable age calibrations from Kumar and Hedges (1998) and because multiple substitutions might have occured at the same sites of the highly divergent mitochondrial sequences. In the latter case, calculated substitution rates would not be expected to be similar between a 100-million-year-old node and nodes that are about 500 million years old, and the observed similarity would therefore not corroborate the assumed age of 100 Ma, but instead indicate that it is overestimated.

Vences et al. (2001)

Vences et al. (2001) used a similar approach to Kumazawa et al. (2000) with a different dataset and came to opposite conclusions. The dataset included mitochondrial sequences of the 16S ribosomal RNA gene ($\sim$520 bp) for 88 cichlid species and nuclear sequences of the Tmo-4C4 locus (Streelman and Karl, 1997; a 511 bp fragment of the titin gene) for 24 cichlids. Substitution rates were calculated separately for the Lake Tanganyika tribes Eretmodini and Lamprologini, the Mbuna and non-Mbuna groups of Lake Malawi, and for species from Lake Barombi Mbo, assuming that all groups had radiated in situ in each lake, that radiation onset occurred with lake formation in each case, and that Lakes Tanganyika, Malawi, and Barombi Mbo are 12, 2, and 1 million years old, respectively. A comparison of averaged 16S substitution-rate estimates to analogous estimates from mammals (bovids, cervids, and equids) showed that these were similar, which was taken as a confirmation of their results by Vences et al. (2001). Substitution-rate estimates were then used to infer divergence times of cichlid subfamilies and indicated that Pseudocrenilabrinae and Cichlinae separated about 78–24 Ma; those authors therefore argued for oceanic dispersal instead of Gondwanan vicariance.

We now know that several of the assumptions of Vences et al. (2001) are questionable: The tribes Eretmodini and Lamprologini appear to have radiated long after the origin of Lake Tanganyika (Meyer et al., 2017), some of the ages assumed for the calculation of mammalian substitution rates were probably wrong by a factor of almost 2 (e.g., the divergence of *Ovis* and *Capra* was assumed at 5 Ma but recently estimated at 9.1 Ma by Toljagić et al., 2018), and similarity in 16S substitution rates of cichlids and mammals may have been coincidental given the large among-clade rate variation of ribosomal DNA (estimated as $4.6 \pm 1.4 \times 10^{-3}$ Myr^{-1}, mean ± standard deviation, by Toljagić et al., 2018). Nevertheless, as rightfully pointed out by Vences et al. (2001) the assumed coincidence of clade radiations and lake formations is expected to lead to conservative age estimates that are rather over- than underestimated.

Genner et al. (2007)

Genner et al. (2007) were the first to apply Bayesian divergence-time estimation to address the question of Gondwanan vicariance. Those authors used two mitochondrial (16S and cytb) and one nuclear (Tmo-4C4) marker, sequenced for 28 cichlid and two outgroup species, for parallel phylogenetic divergence-time analyses that were calibrated either with the cichlid fossil record or according to the Gondwanan vicariance scenario. As these two analyses alone would not allow the assessment of which timeline is more probable, those authors also conducted more extensive analyses in which they fitted cichlid diversification into the timelines of teleost diversification from five independent studies that did not include cichlids (Inoue et al., 2005; Steinke et al., 2006; Yamanoue et al., 2006; Benton and Donoghue, 2007; Hurley et al., 2007). The timelines of the first four of these studies were themselves based on molecular-clock analyses, and they were implemented by Genner et al. (2007) using age estimates from these studies as calibration points in joint analyses of cichlid and non-cichlid 16S and cytb sequences. The reliability of the resulting age estimates for cichlids thus depends on the accuracy of these independent timelines, a discussion of which would be beyond the scope of this review. However, the timeline of the fifth of these studies, by Benton and Donoghue (2007), was not based on sequence data, but on a detailed interpretation of the fossil record; therefore, I will here discuss Genner et al.'s divergence-time estimates resulting from the implementation of this timeline. The timeline of Benton and Donoghue (2007) consisted of minimum and maximum ages for all 40 divergence events in a consensus phylogeny of 41 animal species. To utilize some of this information, Genner et al. (2007) combined published 16S and cytb sequences of five non-cichlid teleost species and one shark species with their own dataset, which allowed them to estimate divergence times of cichlid fishes based on four of the minimum and maximum ages provided by Benton and Donoghue (2007).

Divergence times were estimated with the autocorrelated clock model implemented in the Bayesian-inference software multidivtime (Thorne et al., 1998). The resulting age estimates indicated the divergence of Pseudocrenilabrinae and Cichlinae to be around 98.4 Ma with a 95% highest-posterior-density (HPD) interval from 121.9 to 75.4 Ma, in good agreement with the Gondwanan vicariance scenario. As a consequence, substitution rates estimated in this analysis were similar to those from the analysis calibrated according to Gondwanan vicariance, but disagreed with those from the analysis calibrated with cichlid fossils (Fig. 3 in Genner et al., 2007). The authors therefore argued that "these results, together with evidence that the Gondwanan landmass fragmented in the same chronological order as cichlid phylogenetic reconstructions, support Early Cretaceous cichlid origins."

However, the results based on the timeline of Benton and Donoghue (2007) may have been confounded by incorrect assumptions about the relationships of the six outgroup species, which were fixed for Genner et al.'s analyses with the software multidivtime, and were in conflict with the current understanding of teleost phylogeny: medaka (*Oryzias latipes* (Temminck & Schlegel, 1846)) was placed outside of a clade formed by all other included percomorph taxa even though medaka is a member of Ovalentaria together with cichlids, and tetraodontids were placed next to cichlid fishes even though they are now known to be closer to ninespine stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) (Betancur-R et al., 2017).

As another potential source of bias in the analysis with the Benton and Donoghue (2007) set of calibrations, divergence times of cichlids were probably most directly influenced by the phylogenetically closest calibration, a rather wide constraint (150.9–96.9 Ma) on the divergence of pufferfishes based on the fossil *Plectocretacicus clarae* Sorbini, 1979, from the deposits of Haqil, Lebanon. This fossil was at the time assumed to represent the earliest record of Tetraodontiformes; however, this assignment has since been revised: Instead of *Plectocretacicus clarae*, the fossil *Cretatriacanthus guidotti* Tyler & Sorbini, 1996, from Nardò, Italy, is now more commonly considered to represent the earliest record of Tetraodontiformes (Santini et al., 2013; Dornburg et al., 2014; Friedman, 2014; Benton et al., 2015; Santini, priv. comm.), with a minimum age of 69.7 Ma (Benton et al., 2015) or 83.0 Ma (Chen et al., 2014; Santini, priv. comm.). However, it is worth noting that uncertainty also remains regarding the phylogenetic position of *Cretatriacanthus guidotti*, and it has recently been argued that all representatives of the so-called "plectocretacicoids," including *Plectocretacicus*, *Cretatriacanthus*, as well as two other fossil genera, might be only distantly related to Tetraodontiformes (Alfaro et al., 2018). If this was the case, the earliest appearance of Tetraodontiformes would be even younger than *Cretatriacanthus guidotti*. In addition, the recommended maximum age for this constraint has also been reduced by 25 Myr by Benton et al. (2015) who now suggest to use 130.8 Ma instead of 150.9 Ma. As described in the Discussion, a reanalysis of the sequence data of Genner et al. (2007) with the timeline of Benton and Donoghue (2007), taking into account the correct relationships of outgroups as well as the revised fossil constraint, no longer supports Gondwanan vicariance.

Azuma et al. (2008)

Like Genner et al. (2007), Azuma et al. (2008) also performed Bayesian phylogenetic analyses explicitly to evaluate the Gondwanan vicariance scenario. The molecular dataset used by Azuma et al. (2008) was far larger than those of earlier studies as it contained full mitochondrial genome sequences of 54 taxa, including four species of Pseudocrenilabrinae and two species of Cichlinae. After excluding markers with "poor phylogenetic performance" (Azuma et al., 2008), other unalignable regions, and third-codon positions of coding genes, the alignment contained 10,034 bp. Time calibration was based on a set of 18 constraints of which only the oldest three had minimum and maximum boundaries, for all other constraints only minimum boundaries were specified. This strategy was common in papers from the group of Mutsumi Nishida (e.g., Inoue et al., 2005; Miya et al., 2010) and was explained in Inoue et al. (2005) with the argument that "maxima are intrinsically more difficult to estimate." While maximum boundaries are in fact harder to derive than minimum boundaries (which are simply given by fossil ages), they are absolutely essential for Bayesian divergence-time estimation; without maximum boundaries, age estimates could in principle become infinitely large. Azuma et al. (2008) apparently acknowledged this requirement with the specification of the three maximum boundaries on the oldest divergences.

However, a short thought experiment, illustrated in Fig. 3, is sufficient to reveal that this practice of using maximum ages exclusively for older nodes can easily lead to overestimation of intermediate ages: If we imagine a phylogeny in which only the first divergence is calibrated without error (i.e., the root is fixed to its true age), clock-rate variation would likely lead to some of the other divergences being overestimated and others being underestimated. The addition of minimum boundaries for the overestimated divergences would not have an effect, but constraining the underestimated divergences with minimum boundaries would push these upward. Thus, assuming that early divergences are correctly constrained, the use of only minimum ages for intermediate divergence may be expected to induce bias toward age overestimation.

Moreover, the maximum constraints placed on the first two divergences were, just like those of Kumazawa et al. (2000), taken from the controversial study of Kumar and Hedges (1998) and may thus be considered unreliable (Graur and Martin, 2004). Notwithstanding these potential issues, Azuma et al. (2008) interpreted their estimated timeline, including a divergence time of Pseudocrenilabrinae and Cichlinae at 89.0 Ma (95% HPD 108.0-72.0), as "strong evidence for the vicariant hypothesis."

Santini et al. (2009)

In contrast to Genner et al. (2007) and Azuma et al. (2008), the study of Santini et al. (2009) did not aim to test the Gondwanan vicariance hypothesis. Instead, Santini et al. (2009) used a large and diverse taxon set of bony fishes to estimate shifts in the speciation rate in relation to the teleost-specific whole-genome duplication (Glasauer and Neuhauss, 2014). Nevertheless, the taxon set of Santini et al. (2009) also included six species of Pseudocrenilabrinae and one member of Cichlinae, and the results of this study are thus relevant for the question of Gondwanan vicariance versus trans-Atlantic dispersal. Using the uncorrelated lognormal (UCLN) relaxed-clock model implemented in BEAST (Drummond et al., 2006; Drummond and Rambaut, 2007), Santini et al. (2009) established their timeline of bony fish divergences based on 227 sequences of the nuclear recombination-activating gene 1 (rag1) and 45 fossil calibration points. Unlike in Genner et al. (2007) and Azuma et al. (2008), these fossil calibrations were implemented not merely as minimum and maximum boundaries for divergence times, but with the parametric prior-probability density functions (henceforth referred to as "prior densities") available in BEAST. The advantage of these parametric prior densities is that they allow the specification of "soft" maximum ages (Benton and Donoghue, 2007) which enable a more accurate modeling of the expectation that the probability of a divergence time declines gradually the further it is shifted back in time. On the other hand, the use of parametric prior densities for fossil calibrations does not relieve the user from the need to specify, for each constrained divergence, a maximum age above which she or he considers the divergence time to be extremely improbable.

In Santini et al. (2009), lognormal prior densities were specified for all fossil calibration so that 95% of the probability mass lay within an interval younger than the ages of related taxa in the fossil record. While this practice may be preferred over the specification of prior probabilities without objective criteria (as in Matschiner et al., 2011), it assumes that the related taxa did not co-occur with the constrained lineages, an assumption that may not hold in many cases. In the study of Santini et al. (2009), the practice led to some extremely narrow prior densities, such as for the divergence of Ostariophysi and Clupeomorpha (= Clupei; Betancur-R et al., 2017), for which a minimum age of 149 Ma is given by the fossil *Tischlingerichthys viohli* Arratia, 1997, from the upper Solnhofen Limestone of Bavaria, Germany (Arratia,

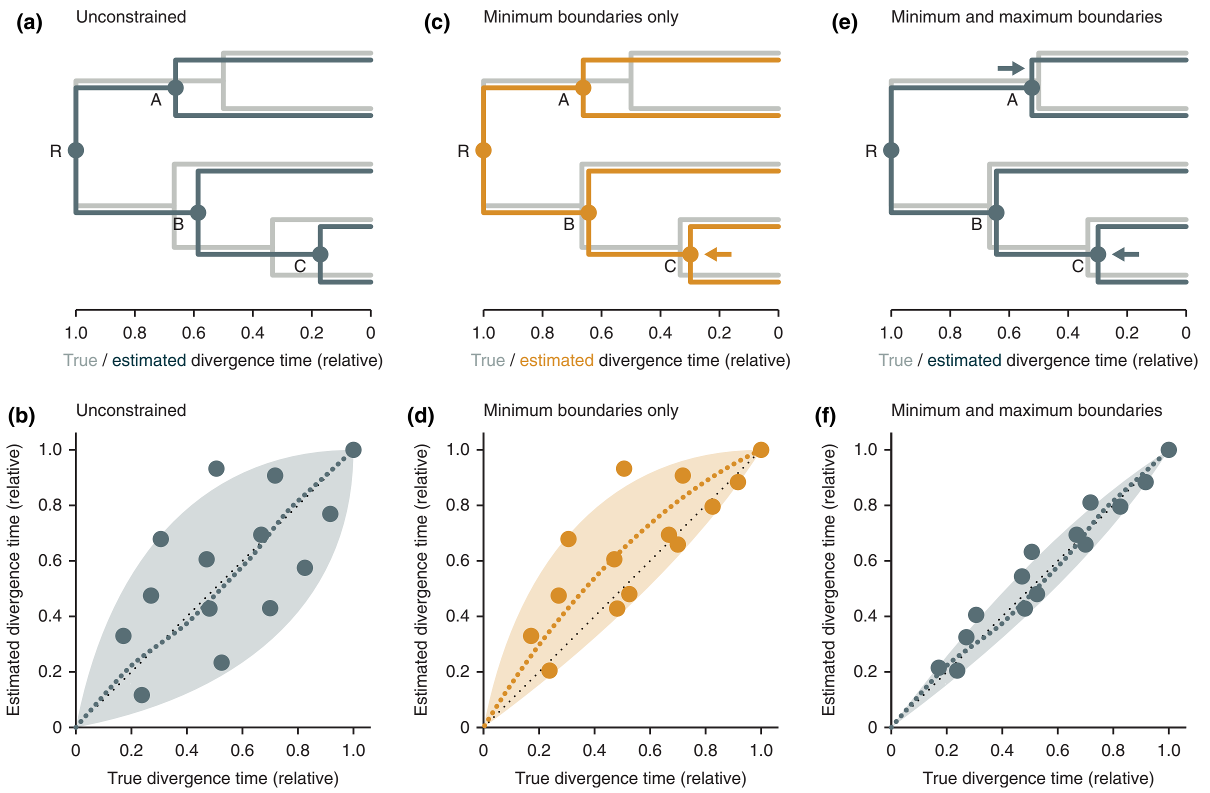

Fig. 3 Expected bias and precision when intermediate divergence times are left unconstrained, when only minimum boundaries are used, or when both minimum and maximum boundaries are applied. **a** Assuming that the root (node R) of a hypothetical phylogeny is fixed to its true age but no other divergences are constrained, clock-rate variation is expected to lead to some divergence times being overestimated (node A) and others (nodes B and C) being underestimated. True divergence times are shown by the phylogeny in light gray; estimated divergence times are marked by the phylogeny in dark gray. In a larger hypothetical phylogeny constrained as in (**a**), estimates of intermediate divergence times are therefore expected to be distributed as shown in (**b**). **b** Individual divergence-time estimates of phylogenies are marked with gray dots. Dots below the diagonal represent underestimated divergence times, and divergence times marked with dots above the diagonal are overestimated. A sliding-window mean estimate is shown as a gray dotted line. This line is close to the diagonal, indicating that divergence-time estimates are overall unbiased, albeit imprecise. **c** The addition of constraints with minimum boundaries only is expected to correct divergence times that were previously underestimated (nodes B and C), but has no effect on overestimated divergence times (node A). In general, this is expected to increase precision but at the same time introduce bias as shown in (**d**). **d** Underestimated divergence times are now closer to the diagonal. However, as a result, the sliding-window mean now lies above the diagonal, indicating biased age estimates especially for intermediate divergence times. **e** The further addition of maximum boundaries is expected to decrease the age estimates for divergences that were previously underestimated (node A). **f** Overestimated divergence times are now also closer to the diagonal. Thus, the addition of maximum boundaries (assuming that these were appropriately applied) has increased precision and at the same time reduced bias, as indicated by the sliding-window mean that is now again close to the diagonal. The color used in (**c**) and (**d**) indicates that this expectation applies to the divergence-time estimates of Azuma et al. (2008) shown in Figs. 2, 5 and 6. However, note that this expected bias has not yet been confirmed with simulations

1997). A soft maximum age was derived from the occurrence of elopomorph fossils from deposits that were merely three million years older; thus, 95% of the prior-probability mass lay between 152 and 149 Ma and half the probability mass was concentrated within 1.1 Myr. It may be questioned whether the existence of these elopomorph fossils (which in fact are only very distantly related to the constrained lineages) really gives us a 95% confidence that the divergence of Ostariophysi and Clupeomorpha must lie within these three million years.

The age of *Mahengechromis* was used as a constraint in the analysis of Santini et al. (2009); however, *Mahengechromis* was only assumed to be a

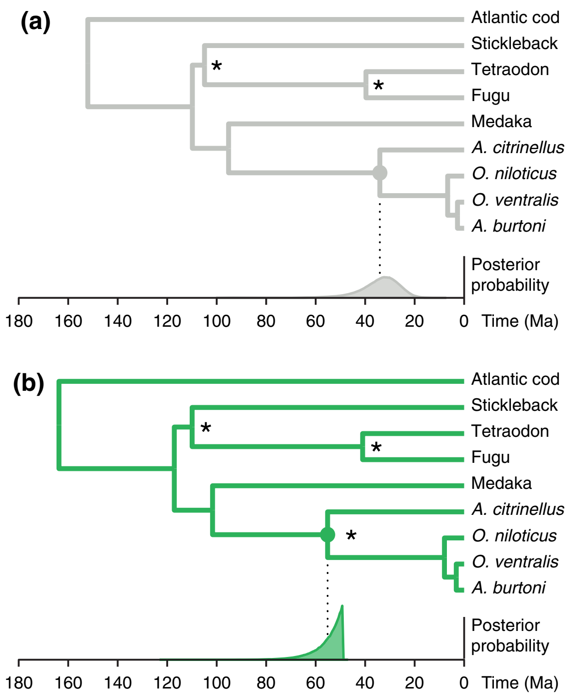

Fig. 4 Divergence-time estimates resulting from analyses of transcriptome data (Matschiner, unpublished). **a** Results without the use of cichlid fossil constraints. **b** Results after adding a constraint based on the age of fossils from the Lumbrera Formation (which was assumed to be 49 Ma in Matschiner, unpublished). Calibration points are marked with asterisks. Below both plots, the posterior-probability density for the divergence time of Pseudocrenilabrinae and Cichlinae is shown as a gray outline. The color used in (**b**) indicates that this result corresponds to the one reported for Matschiner (unpublished) in Figs. 2, 5 and 6

member of the family Cichlidae and not, as suggested by Murray (2001b), considered as the oldest fossil record of Pseudocrenilabrinae with a tentative sister-group relationship to *Hemichromis*. The divergence of Pseudocrenilabrinae and Cichlinae was instead constrained with a minimum of 23.3 Ma, the age of fossils putatively assigned to the extant genus *Heterochromis* (Lippitsch and Micklich, 1998; see Introduction). The resulting divergence-time estimate for Pseudocrenilabrinae and Cichlinae was markedly younger than in the studies of Genner et al. (2007) and Azuma et al. (2008), with a mean estimate of 49.0 Ma and a 95% HPD interval of 66.0–37.0 Ma. While this age estimate provides some support for trans-Atlantic dispersal of cichlids, it is likely that it would have been older if less narrow prior densities had been specified and if the age of *Mahengechromis* had been used to constrain the divergence of Pseudocrenilabrinae and Cichlinae directly.

Matschiner et al. (2011)

While the primary goal of Matschiner et al. (2011) was the estimation of divergence times in notothenioid fishes, our phylogenetic dataset also included four species of Pseudocrenilabrinae and two representatives of Cichlinae. Initially, we included these taxa to provide an age constraint according to the Gondwanan vicariance scenario. However, since cross-validation of ten calibration points using a leave-one-out procedure (Near et al., 2005) indicated conflict between the Gondwanan vicariance calibration and other constraints, we excluded this calibration and finally estimated cichlid divergences on the basis of six internally consistent calibrations, for which we specified relatively wide prior densities with a mix of hard and soft maximum ages. The dataset used for these analyses was composed of two mitochondrial and four nuclear markers (4,599 bp), sequenced for 83 species of acanthomorph fishes.

Divergence times were estimated with the software BEAST, employing the UCLN relaxed-clock model. The results of our final analyses suggested that Pseudocrenilabrinae and Cichlinae diverged around 59.7 Ma, with a 95% HPD interval ranging from 80.7 to 40.2 Ma. However, these age estimates should be viewed with caution due to the small number of cichlid species analyzed and the use of rather few fossil calibrations. Moreover, the widths of the lognormal prior densities specified for these calibrations were selected mostly based on intuition rather than objective criteria, a problem that affected most Bayesian dating studies at the time. Even though objective approaches suitable for the definition of prior densities had been developed as early as 1997 by paleontologists (Marshall, 1997; Marshall, 2008; Hedman, 2010), these were not widely known among practitioners of molecular dating, and the use of such approaches became widespread only later with the development of methods that were more explicitly designed for molecular studies (Nowak et al., 2013; Heath et al., 2014; Matschiner et al., 2017).

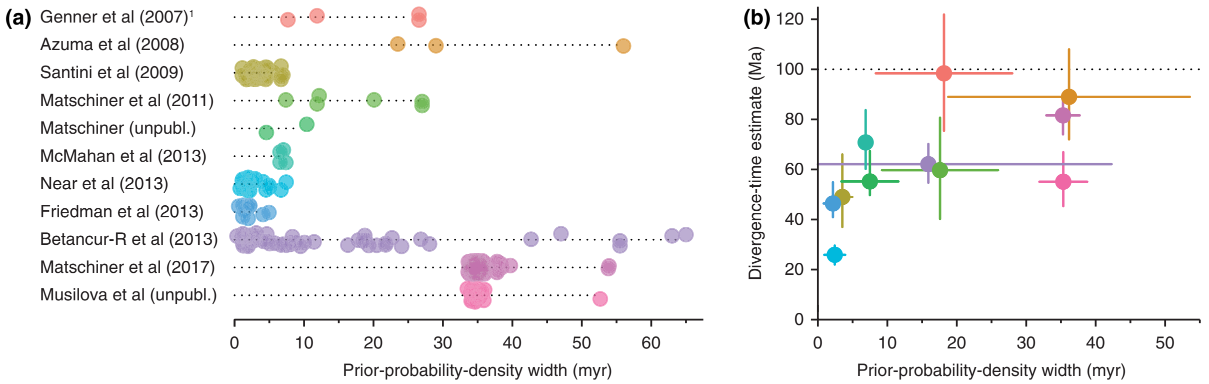

Fig. 5 Prior densities used for fossil constraints in eleven Bayesian dating studies. **a** For each fossil constraint, the width of the corresponding prior density is plotted. These widths are here measured as the length of the interval, beginning with the age of the fossil, that contains 50% of the prior-probability mass. **b** Comparison of the prior-density widths used in the eleven studies with the resulting age estimates for the divergence of Pseudocrenilabrinae and Cichlinae. Error bars indicate standard deviations of prior-density widths on the horizontal axis and 95% HPD density intervals for divergence-time estimates on the vertical axis. Color code in (**a**) and (**b**) is identical to Figs. 2, 3, 4 and 6. [1]Only the analysis based on the Benton and Donoghue (2007) set of calibrations is shown for Genner et al. (2007)

Matschiner (unpublished)

The results of Matschiner et al. (2011) led me to explore divergence times of cichlids further with a complementary dataset that had become available in the same year and included only few species but a much larger number of markers. The basis of this dataset were the transcriptomes of *Astatotilapia burtoni* (Günther, 1894), *Ophthalmotilapia ventralis* (Boulenger, 1898), and *Oreochromis niloticus* (Linnaeus, 1758) (all Pseudocrenilabrinae), *Amphilophus citrinellus* (Günther, 1894) and *A. zaliosus* (Barlow, 1976) (Cichlinae), as well as of medaka (*Oryzias latipes*), published by Elmer et al. (2010) and Baldo et al. (2011). Through BLAST (Altschul et al., 1990) searches with a strict similarity threshold, I identified orthologous sequences in the proteomes of ninespine stickleback (*Gasterosteus aculeatus*), fugu (*Takifugu rubripes* (Temminck & Schlegel, 1846)), tetraodon (*Dichotomyctere nigroviridis* (Marion de Procé, 1822), and Atlantic cod (*Gadus morhua* Linnaeus, 1758), that were all available in the ENSEMBL database (Flicek et al., 2011). This procedure resulted in a set of 126 orthologous markers (28,239 bp).

For divergence-time estimation, two fossil calibrations were used with prior densities as recommended by Benton and Donoghue (2007): the divergence of fugu and tetraodon was constrained by the oldest member of Tetraodontidae, *Archaeotetraodon winterbottomi* Tyler & Bannikov, 1994, with an age estimate of 32.25 Ma (Santini and Tyler, 2003). In addition, *Plectocretacicus clarae* was assumed to be the oldest representative of the order Tetraodontiformes with a minimum age of 96.9 Ma (Tyler and Sorbini, 1996) and thus constrained the divergence between ninespine stickleback and the two tetraodontids. Finally, after initial analyses resulted in an extremely young estimate for the divergence of Pseudocrenilabrinae and Cichlinae (34.1 Ma; 95% HPD 50.2–20.1 Ma) that was in conflict with the fossil record of cichlids (Fig. 4), this divergence was further constrained to be older than 49.0 Ma (which at the time was the assumed age of fossils from the Lumbrera Formation; Malabarba et al., 2010). After this adjustment, the divergence of Pseudocrenilabrinae and Cichlinae was estimated at 55.2 Ma with a 95% HPD interval from 67.4 to 49.9 Ma.

Just like the study of Matschiner et al. (2011), these unpublished results should be viewed with caution due to the small number of taxa and fossil constraints. Nevertheless, it is remarkable how the large nuclear dataset used in this study seemed to “pull” cichlid divergence times toward extremely young ages unless this tendency is counteracted by the specification of cichlid fossil constraints. Moreover, it appears that this tendency applies only to cichlid divergence times, not to those of the outgroups, given that the age estimates of the separation of the Atlantic cod (163.7 Ma; 95%

HPD 224.1–109.7 Ma) and the divergence of nine-spine stickleback and tetraodontids from medaka and cichlids (117.1 Ma; 95% 141.3–100.2 Ma) were very similar to those of newer phylogenomic studies (150.9 Ma and 115.3 Ma, respectively, in Malmstrøm et al., 2016). It may be speculated that a slow-down of the nuclear substitution rate in cichlids is at least partially responsible for the extremely young divergence-time estimates without cichlid fossil constraints (Fig. 4a); however, because estimates of rates and ages are confounded, reliable rate estimates will only be available after a robust timeline of cichlid diversification has been established.

McMahan et al. (2013)

McMahan et al. (2013) generated a time-calibrated phylogeny of cichlids primarily to investigate whether speciation-rate shifts have occurred throughout their evolutionary history, but conclusions were also drawn regarding Gondwanan vicariance. While the molecular dataset of McMahan et al. (2013) was comprehensive in terms of taxon coverage (132 species, including 19 species of Pseudocrenilabrinae and 57 species of Cichlinae), only two mitochondrial and two nuclear markers (2,069 bp) were used in their analysis. Divergence-time estimation was performed with the UCLN relaxed-clock model, calibrated with three constraints on cichlid divergences and a single constraint outside of cichlids. The three constraints within cichlids were based on the ages of *Gymnogeophagus eocenicus* and *Plesioheros chauliodus* (both assumed to be 40 Ma) as well as *Mahengechromis*. The calibration outside of cichlids was placed on the divergence of the outgroup *Polymixia lowei* Günther, 1859; this divergence was constrained with the "minimum age of 95 Ma [...], based on the fossil taxon *Polymixia* sp. known from Middle-Upper Cenomanian deposits." Even though no fossil taxon of this name is known or listed in the cited reference (Patterson, 1993) the constraint is not misplaced because several fossils with a similar age were in fact assigned to Polymixiiformes (reviewed in Supplementary Text S2 of Matschiner et al., 2017). Based on these four constraints, McMahan et al. (2013) estimated the divergence of Pseudocrenilabrinae and Cichlinae at 70.8 Ma with an 95% HPD interval ranging from 83.7 to 60.2 Ma.

The age of the most recent common ancestor of cichlids was estimated at 81 Ma (95% HPD 96–67 Ma), which the authors claim to be "consistent with previous Gondwanan vicariance hypotheses that have explained the present distribution of cichlid taxa" (McMahan et al., 2013); however, this is clearly not the case. Instead, any divergence timeline consistent with Gondwanan vicariance would necessarily place the common ancestor of cichlids before 120 Ma and possibly as early as 150 Ma (see Introduction).

Near et al. (2013)

Shifts in diversification rates were also investigated by Near et al. (2013), albeit in a much larger context than by McMahan et al. (2013). Near et al. (2013) tested for rate variation across the acanthomorph phylogeny, which they estimated based on ten nuclear markers sequenced for 579 species. The dataset of Near et al. (2013) is thus the largest among the studies compared here, and it ranks among the largest ever to be analyzed with BEAST. However, as the study was not focused on cichlids, only two species of Pseudocrenilabrinae and three species of Cichlinae (as well as two representatives of Ptychochrominae and two of Etroplinae) were included in the dataset, no cichlid fossils were used for calibration, and divergence times of cichlids were not discussed (even though Pseudocrenilabrinae and Cichlinae were together identified as a clade with elevated speciation rates). Nevertheless, owing to the large dataset and the use of as many as 37 fossil calibrations, the resulting timeline of acanthomorph evolution might be expected to be rather reliable. Importantly, the prior densities used for these calibrations were defined according to guidelines laid out by paleontologists (Benton and Donoghue, 2007; Marshall, 2008). While these guidelines are in turn based on assumptions that may not be met in many cases, the use of such objective criteria is certainly an improvement over the previously common practice of specifying prior densities based on intuition (e.g., Matschiner et al., 2011). On the other hand, as in Santini et al. (2009), many of the calibrations were characterized by very narrow prior densities; an extreme case of this is the divergence of genera *Archoplites* and *Ambloplites* within sunfishes (Centrarchidae) that was constrained so that 95% of the prior-probability mass lay between 15.5 and 17.8 Ma. Unfortunately, Near et al. (2013) provide no details for how these prior densities were calculated, except that the FA_{95} method of Marshall (2008) was used.

Possibly as a result of the narrow prior densities, the divergence of Pseudocrenilabrinae and Cichlinae was estimated at an extremely young age of 25.9 Ma, with a 95% HPD interval ranging only from 29.6 to 22.0 Ma. Thus, a literal interpretation of these results would lead us to reject an origin of Cichlinae before 30 Ma. While these divergence-time estimates cannot be taken at face value, particularly because the cichlid fossil record was ignored, they suggest that if *Mahengechromis* had been used for calibration, the resulting divergence-time estimate for Pseudocrenilabrinae and Cichlinae would not have been much older than *Mahengechromis* itself, 46–45 Ma.

Friedman et al. (2013)

Friedman et al. (2013) expanded on the study of Near et al. (2013) with a molecular dataset containing sequences of the same ten nuclear markers for many more cichlid species, with the goal of explicitly testing the Gondwanan vicariance hypothesis. In total, their dataset contained 158 species, including 54 species of Pseudocrenilabrinae, 29 species of Cichlinae, and three species from each of Etroplinae and Ptychochrominae. Divergence times were estimated with ten fossil constraints, all of which were directly copied from Near et al. (2013). Presumably, the remaining 27 calibrations of Near et al. (2013) could not be used due to the reduced dataset. Notably, Friedman et al. (2013) also did not add any calibrations within cichlids "so that our [...] estimate of the evolutionary time scale for the group is truly independent of its fossil record" (Friedman et al., 2013). Furthermore, eight of the ten calibrations copied from Near et al. (2013) also share narrow prior densities that have 95% of the probability mass within less than 10 Myr. The mean divergence-time estimate for Pseudocrenilabrinae and Cichlinae, 46.4 Ma, is consistent with the fossil record of cichlids; however, the confidence interval for this estimate is not (95% HPD 54.9–40.9 Ma) as it extends to ages younger than *Mahengechromis*. Moreover, diversification within Cichlinae only begins at about 29.2 Ma (95% HPD 34.8–25.5 Ma) in the timeline of Friedman et al. (2013), which disagrees with the nested positions of the Lumbrera Formation fossils that indicate that the subfamilies Heroini and Geophagini, and probably the genus *Gymnogeophagus* originated before 40 Ma (Malabarba et al., 2010; Perez et al., 2010). Nevertheless, the results of Friedman et al. (2013) are in strong conflict with the assumption of Gondwanan vicariance; as a consequence, the authors argued that cichlid biogeography can only be explained by marine dispersal.

The authors further corroborated their claim with two separate paleontological analyses. In the first of these analyses, they compiled a database of fish-bearing freshwater deposits with ages up to around 200 Ma and concluded that cichlid fossils older than 46 Ma should have long been found if cichlids really had existed much earlier (before 77.8 Ma). Concordant with this were the results of the second paleontological analysis, a comparison of fossil ages from successive outgroups of cichlids following Hedman (2010): This comparison indicated that cichlids originated at about 60.7–57.0 Ma, with confidence intervals ranging from 90.1 to 46.8 Ma.

Betancur-R et al. (2013)

Betancur-R et al. (2013) compiled a massive molecular dataset of 20 nuclear and one mitochondrial marker for as many as 1 410 species (albeit with 63% missing data), to establish a new classification of bony fishes. While the full dataset was used for maximum-likelihood phylogenetic analyses, a reduced version of it (18 markers for 202 species with 48.5% missing data) was also used for Bayesian divergence-time estimation with the software BEAST. Time calibration was based on 58 fossil calibrations, of which 30 were taken from Near et al. (2012) and thus also had very narrow prior densities (17 of the calibrations contained 95% of the probability mass within less than 10 Myr). Fossils from the Lumbrera Formation were assumed to date to 49 Ma (López-Fernández et al., 2013) and were used to constrain the ages of Geophagini and Heroini. In addition to the 58 fossil calibrations, a biogeographic calibration was used: assuming that the final closure of the Isthmus of Panama was causal for the separation of Atlantic and Indo-Pacific trumpetfishes (Bowen et al., 2001), their divergence was constrained to 3.5–2.8 Ma. Given that recent research suggested that many species pairs now separated by the isthmus may have diverged millions of years before its final closure, this age might be an underestimate (Bacon et al., 2015; Stange et al., 2018). The age of Tetraodontiformes was constrained, but in contrast to Genner et al. (2007) and Matschiner (unpublished), Betancur-R et al. (2013) considered *Cretatriacanthus*, with an assumed age of 85 Ma, to represent the earliest tetraodontiform record, which is likely more appropriate (Chen et al., 2014; Santini, priv. comm.). The divergence of Pseudocrenilabrinae and Cichlinae was estimated at 62.1 Ma with a 95% HPD interval ranging from 70.2 to 54.7 Ma.

Matschiner et al. (2017)

Realizing that the often arbitrarily defined shapes of prior densities for fossil constraints can have tremendous impact on divergence-time estimates (see Brown and Smith, 2017, for a recent discussion of this issue) and thus on the conclusions of dating studies, a primary goal of Matschiner et al. (2017) was the development of a more quantitative approach for the specification of these densities. To this end, we assumed a model of time-homogeneous diversification and fossilization processes, from which prior densities for clade ages could directly be derived given the age of the oldest fossil record of each clade. We implemented our approach in the CladeAge add-on package for the software BEAST 2 (Bouckaert et al., 2014). The great advantage of this approach is that it relieves the user from the need to choose the shape of prior densities for each fossil constraint; however, it requires estimates of diversification and fossil-sampling rates instead, and may generate biased results if these rates are misspecified. A further requirement is that strictly all clades are calibrated that (i) are morphologically recognizable, (ii) have a fossil record, and (iii) have their sister lineage included in the phylogeny; the latter criterion ensures that the beginning of the branch leading to the clade actually represents the origin of the clade. While the sister lineage of a clade may often not be known before the analysis, its inclusion can be guaranteed when a so-called “diversity tree” (Alfaro et al., 2009) is used for the analysis, in which each extant species of a group can be assigned to one tip of the phylogeny (a simple example would be a phylogeny with one sampled species for each genus of a family). Thus, to maximize the utility of our approach for divergence-time estimation in cichlids, we compiled a large dataset that would allow the generation of a diversity tree for the group of Clupeocephala (= all teleost fishes except the most ancient lineages Elopomorpha and Osteoglossomorpha; Betancur-R et al., 2017).

Our large taxon set with 366 terminal lineages allowed us to specify as many as 147 age constraints, using our newly developed approach. Prior densities for each of these constraints were based on an assumed net diversification rate of 0.041–0.081 Myr^{-1}, a turnover of 0.0011–0.37, and a fossil-sampling rate of 0.0066–0.01806 Myr^{-1} (Foote and Miller, 2007; Santini et al., 2009), taking into account the uncertainties in these estimates as well as in the fossil ages. However, the use of our large taxon set came at the cost of a rather sparse data matrix because we had to include several species for which only one or few mitochondrial sequences and no nuclear sequences were available. As a consequence, our dataset contained close to 60% missing data (Table 1) and was dominated by mitochondrial sequences. Our results suggested that Pseudocrenilabrinae and Cichlinae diverged about 81.6 Ma, with a 95% HPD interval from 89.4 to 74.0 Ma. Importantly, this age estimate was driven by the molecular sequence data rather than the specified prior distributions, as shown by an additional analysis based on the prior alone that resulted in a mean divergence time greater than 100 Ma.

Even though the large number of fossil constraints, together with their model-based prior densities, suggests that the timeline presented in Matschiner et al. (2017) may be rather reliable, the divergence time of Pseudocrenilabrinae and Cichlinae might still be overestimated for two reasons: First, the model used to specify prior densities for fossil calibrations assumed constant diversification and fossilization, which may rarely be met in nature. If instead these processes vary over time and among the investigated taxa (there is plenty of evidence for both; e.g., Near et al., 2013; Wagner and Marcot, 2013), some prior densities used in our study would have been too narrow while others would have been too wide. The combined effect of both would likely lead to age overestimation as narrowing these densities may have more impact than widening. Second, as our simulations in Matschiner et al. (2017) have shown, autocorrelation of substitution rates may also lead to age overestimation with our approach and is likely present among the taxa used in our study. In summary, we argued in Matschiner et al. (2017) that “our analyses strongly support trans-Atlantic dispersal of cichlid fishes."

Musilova et al. (unpublished)

I further applied the calibration approach developed for Matschiner et al. (2017) in a yet-to-be-published study by Musilova et al., using whole-genome data of 100 teleost species and one non-teleost outgroup. The pipeline used for the identification of suitable phylogenetic markers was similar to that used in Malmstrøm et al. (2016) and led to an alignment containing sequences of 113 nuclear genes with a total length of 71,902 bp. Due to the lower number of taxa included in this study, only 28 fossil constraints, a fraction of those used in Matschiner et al. (2017), could be applied. Owing to the focus on species with available genomic data, also only a single representative of Cichlinae (*Amphilophus citrinellus*) was included in the analysis. As a consequence, the nested positions of *Proterocara argentina*, *Plesioheros chauliodus*, and *Gymnogeophagus eocenicus* could not be taken into account, and instead the Lumbrera Formation fossils were only used to constrain the age of Cichlinae as a whole. Similarly, as no member of Hemichromini was included, *Mahengechromis* was not used to constrain the divergence of the tribe, as tentatively suggested by (Murray 2000b; see above), but instead only served as the oldest record of Pseudochrominae.

The resulting timeline suggested that Pseudocrenilabrinae and Cichlinae diverged 55.1 Ma, with a 95% HPD interval ranging from 66.9 to 45.3 Ma. Given that this confidence interval borders on the age of the earliest cichlid fossil record, it may be assumed that the age estimate would have been older if the cichlid fossils would have been placed in the more nested positions suggested by Murray (2000b), Smith et al. (2008), Perez et al. (2010), and Malabarba et al. (2010).

Discussion

The reliability of molecular divergence-time estimates

The comparison of results from eleven Bayesian dating studies reveals great differences in the estimated timelines of cichlid diversification (Table 1). For example, the divergence of Pseudocrenilabrinae and Cichlinae was estimated at 98.4 Ma in Genner et al. (2007), but only about a fourth as old, 25.9 Ma, in Near et al. (2013). Even though the degree of this discrepancy may be surprising, the observation of differences is not unexpected, given that the eleven studies used different datasets, different calibration points, and different prior densities for these calibrations. It should be noted that in a Bayesian context, the use of different datasets alone should not lead to different results (i.e., the confidence intervals should overlap) as long as the data conforms to model expectations and the priors are not grossly misspecified. However, the studies resulting in the most discordant age estimates also used the most contrasting prior specification, suggesting that these differences in priors are in fact a dominant cause of the disagreement in the results. This is illustrated in Fig. 5, which shows that prior densities used in Santini et al. (2009), Near et al. (2013), and Friedman et al. (2013), the three studies resulting in the youngest divergence-time estimates, were extremely narrow compared to other studies. In Near et al. (2013) and Friedman et al. (2013), 50% of the prior-probability mass was on average contained within intervals of only 2.0–2.5 Myr, implying that the authors assumed with great confidence that the origins of the constrained clades could not have predated the ages of the oldest fossils of these clades by more than a few million years. Since 22 out of the 59 fossil constraints used by Betancur-R et al. (2013) were copied from Near et al. (2013), some of the prior densities in Betancur-R et al. (2013) are also extremely narrow. In contrast, comparatively wide prior densities were used for fossil constraints in the two studies resulting in the oldest divergence-time estimates, those of Genner et al. (2007) and Azuma et al. (2008).

So, given that different studies used such extremely different widths for prior densities and that these apparently have a strong influence on divergence-time estimates (Fig. 5; Brown and Smith, 2017), how wide should prior densities ideally be? This is exactly the question that we aimed to address with our model-based approach for prior densities in Matschiner et al. (2017). Our simulations based on a model of constant diversification and fossilization processes suggested that with a probability of around 50%, newly originated clades can remain without a fossil record for about 35 Myr (for this reason the prior densities used in Matschiner et al., 2017, and Musilova et al., unpublished, have a width of around 35 Myr; Fig. 5). While the length of this interval suggests that prior-density widths of around 2.5 Myr are likely too narrow, there are reasons why narrow prior densities may sometimes be justified. For example, our simulations did not take into account that different clades diversify at different speeds and that some are more prone to fossilization than others. Thus, for a rapidly radiating clade with a large number of fossils we can be more confident that clade origin did not predate the age of the oldest fossil by a long period of time. Another justification for narrow prior densities could be the existence of fossil deposits that would be expected to yield members of a certain clade if the clade had existed at the time (e.g., because the location and the environment of the deposits matches those inhabited by the clade). Finally, our simulations did not take into account that some clades may have been selected as calibration points because of other information indicating that their fossil record is relatively old, which would justify the use of narrower prior densities. This could be the case if for example the sister group of the clade is known and has a younger fossil record than the clade used for calibration. Some or all of the above considerations may have contributed to the decisions of study authors to place narrow prior densities on particular clades. In contrast, when using prior densities resulting from our model-based approach, all clades with fossil records should be calibrated regardless of other information about the relative age of their fossil record (which could come from a comparison with the sister group) to avoid bias (Matschiner et al., 2017). For this reason, the prior-density widths used in Matschiner et al. (2017) and Musilova et al. (unpublished) are not fully comparable to those of other studies shown in Fig. 5 and narrower densities can be more appropriate when clades are specifically chosen as calibration points. Nevertheless, it appears unlikely that all of the extremely narrow prior densities of Santini et al. (2009), Near et al. (2013), and Friedman et al. (2013) can be justified in this way, which therefore suggests that the divergence times reported in these studies were at least partially underestimated.

However, Fig. 5b also shows that the different widths of prior densities for fossil constraints can only explain part of the differences in the resulting divergence-time estimates. For example, Matschiner et al. (2011) used very similar calibrations to Genner et al. (2007), and the same is true for Matschiner et al. (2017) and Musilova et al. (unpublished). Nevertheless, the age estimates for the split of Pseudocrenilabrinae and Cichlinae differ by around 40 Myr between Genner et al. (2007) and Matschiner et al. (2011) and by around 25 Myr between Matschiner et al. (2017) and Musilova et al. (unpublished). In the case of Genner et al. (2007) and Matschiner et al. (2011), the difference might partially be explained by stochastic rate variation in the relatively small dataset of Genner et al. (2007), containing sequences of only two markers, and only six non-cichlid species. In addition, Genner et al.'s analysis based on the Benton and Donoghue (2007) timeline might have been influenced by incorrect assumptions for the relationships of the six outgroup taxa, which were fixed in this analysis (see the section on Genner et al., 2007, above). To test whether these incorrect assumptions might have influenced the age estimates in this analysis of Genner et al. (2007), I reanalysed their dataset with the software BEAST 2, using the same age constraints from Benton and Donoghue (2007) but enforcing relationships of outgroup taxa according to the classification of Betancur-R et al. (2017). This reanalysis resulted in a divergence-time estimate of Pseudocrenilabrinae and Cichlinae by around 82.0 Ma, with a 95% HPD interval ranging from 100.4 to 65.5 Ma. While this new estimate is around 16 Myr younger than Genner et al.'s original estimate, the confidence intervals of the two analyses still overlap (the original 95% HPD interval ranged from 121.9 to 75.4 Ma). However, given that my reanalysis was conducted with the software BEAST 2 while the original analysis by Genner et al. (2007) was performed with multidivtime, I cannot rule out that other differences in the analysis settings are responsible for the reduced age estimate, such as my use of the UCLN clock model in BEAST 2, whereas Genner et al. (2007) had used an autocorrelated clock model in their analysis with multidivtime.

In another reanalysis, I also tested how the revised taxonomic assignment of the fossil *Plectocretacicus clarae* (see the section on Genner et al., 2007) might affect the timeline supported by the dataset of Genner et al. (2007). Following Chen et al. (2014) and Benton et al. (2015), I changed the boundaries of the constraint on the origin of Tetraodontiformes from 150.9 to 97.8 Ma (Benton and Donoghue, 2007) to

130.8–83.0 Ma while keeping all other settings identical. As expected, the resulting age estimates are even younger: The divergence of Pseudocrenilabrinae and Cichlinae was estimated at 79.8 Ma, with a 95% HPD interval from 94.1 to 63.4 Ma. However, since Matschiner et al. (2011) used the constraint based on *Plectocretacicus clarae* in the same way as Genner et al. (2007), the old age used for this constraint does not explain the different age estimates of the two studies. As described above, this difference is more likely driven by stochastic variation and the incorrect outgroup relationships used in Genner et al. 's analysis.

Just like with the two studies of Genner et al. (2007) and Matschiner et al. (2011), different age estimates were also obtained by Matschiner et al. (2017) and Musilova et al. (unpublished) despite the use of very similar prior densities for fossil constraints (Fig. 5b). The main difference between the latter two studies was in the datasets used for divergence-time estimation: The study of Musilova et al. (unpublished) included 101 species of which only six were cichlids while Matschiner et al. (2017) included 366 taxa of which nearly half (152) were cichlids. Because of the extensive taxon sampling, Matschiner et al. (2017) were able to place cichlid fossil constraints on the nested branches to which these fossil were assigned taxonomically (within Hemichromini, Geophagini, and Heroini; see Introduction) while the same fossils served only to constrain the divergence of Pseudocrenilabrinae and Cichlinae in Musilova et al. (unpublished); this may have led to age underestimation in the latter study.

On the other hand, the datasets of the two studies also differed strongly in the type of the sequence data: Musilova et al. (unpublished) used a nearly complete alignment of 71,902 nuclear sites, whereas the dataset of Matschiner et al. (2017) consisted of 12,293 mitochondrial and 15,357 nuclear sites. Moreover, the mitochondrial part of the dataset of Matschiner et al. (2017) was more complete (57.8%) than the nuclear fraction of the dataset (34.2% complete), meaning that overall, the dataset of Matschiner et al. (2017) was dominated by mitochondrial data. It is conceivable that the more rapidly evolving mitochondrial sequence data reach an appreciable level of saturation over the hundreds of millions of years of divergence investigated in these studies. As a result, genetic distance would not increase linearly with divergence time, which could bias divergence-time estimates so that particularly divergences of intermediate ages appear overestimated when older divergence times are constrained through fossil calibrations. In fact, a comparison of uncorrected genetic distances to Tamura-Nei distances (Tamura and Nei, 1993) suggests that a substantial degree of saturation is indeed present in the mitochondrial data of Matschiner et al. (2017) (Fig. 6a). In contrast, a

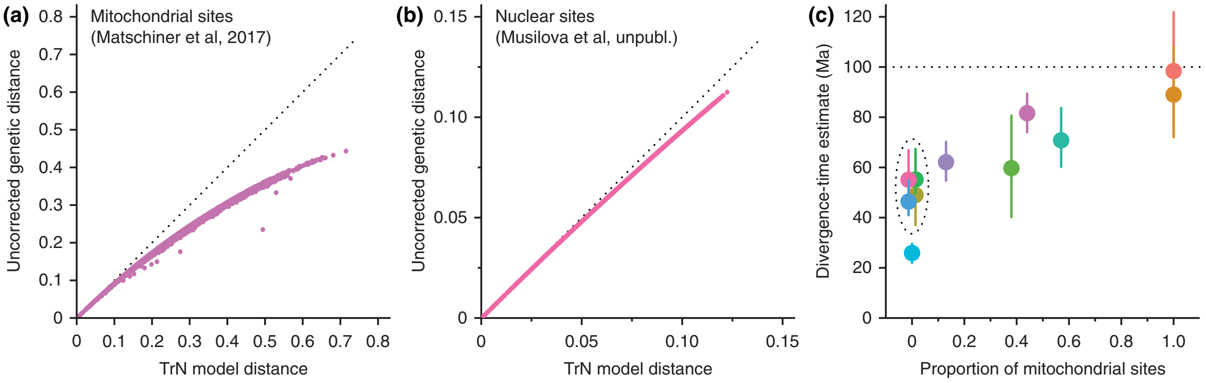

Fig. 6 Saturation in mitochondrial sequences and its effect on divergence-time estimates. **a** Comparison of uncorrelated genetic distances with Tamura-Nei (TrN; Tamura and Nei, 1993) distances for mitochondrial data of Matschiner et al. (2017). Saturation is indicated by the nonlinear correlation of the two distance measures. **b** As (**a**) but for nuclear data of Musilova et al. (unpublished). **c** Comparison of the proportion of mitochondrial alignment sites with the resulting divergence-time estimate for Pseudocrenilabrinae and Cichlinae in the eleven Bayesian dating studies (see Table 1). Error bars indicate 95% HPD density intervals for divergence-time estimates. The dotted ellipse marks two pairs of studies that are displayed side-by-side for better visualization; all four of these studies used no mitochondrial data. The color code indicates the studies as in Figs. 2, 3, 4 and 5

much lower level of saturation is apparent for the nuclear data of Musilova et al. (unpublished) (Fig. 6b). Whether saturation in fact leads to overestimation of intermediate divergence times may depend on how calibration points are placed in a phylogeny, and how it may have affected studies of cichlid divergence times remains to be tested with simulations. Nevertheless, it is remarkable that the five studies resulting in the youngest divergence-time estimates (Santini et al., 2009; Friedman et al., 2013; Matschiner et al, unpublished; Musilova et al., unpublished; Near et al., 2013) were all based exclusively on nuclear data, whereas the two oldest age estimates were obtained with datasets containing only mitochondrial sequences (Genner et al., 2007; Azuma et al., 2008). The apparent correlation between the proportion of mitochondrial sites and the resulting age estimate for the divergence of Pseudocrenilabrinae and Cichlinae in the eleven Bayesian dating studies (Fig. 6c) suggests that saturation in mitochondrial sequences might in fact have led to age overestimation in several studies.

In addition to the issues discussed above, age estimates for cichlid divergences might be biased for further reasons. For example, none of the eleven Bayesian dating studies accounted for incomplete lineage sorting, which is both known to be present in cichlid fishes (Koblmüller et al., 2010) and to influence divergence-time estimates toward overestimation (Meyer et al., 2017; Ogilvie et al., 2017; Stange et al., 2018). Similarly, none of the studies accounted for the possibility that some of the investigated markers might have introgressed between species, which is also common among cichlid fishes (Meier et al., 2017; Meyer et al., 2017) and would presumably lead to age underestimation. However, while both of these processes should ideally be taken into account in future studies, they are likely to bias divergence times by no more than a few million years, and their effect might thus be minor on the timescales relevant for the early cichlid divergences.

In summary, most if not all of the eleven Bayesian dating studies are probably subject to bias due to prior misspecification or model violations. As discussed above, the most significant sources of bias may be extremely narrow prior densities (Santini et al., 2009; Betancur-R et al., 2013; Friedman et al., 2013; Near et al., 2013), a small number of cichlid taxa that prevents the correct placement of fossil calibrations (Matschiner, unpublished; Near et al., 2013; Musilova et al., unpublished), and saturation in mitochondrial sequence data (Genner et al., 2007; Azuma et al., 2008; Matschiner et al., 2017). If these factors in fact act as expected, this would imply that divergence times estimated by Santini et al. (2009), Matschiner (unpublished), Near et al. (2013), Friedman et al. (2013), and Musilova et al. (unpublished) are underestimates while those reported by Genner et al. (2007), Azuma et al. (2008), and Matschiner et al. (2017) are overestimates. As a consequence, one might expect that the true divergence time of Pseudocrenilabrinae and Cichlinae lies in between these dates, which would point to the period between 75 and 60 Ma.

Marine dispersal of cichlid fishes

If Pseudocrenilabrinae and Cichlinae should in fact have diverged around 75–60 Ma, this would mean that they had to traverse the Atlantic at a time when the ocean was already at least 650–900 km wide, about a third of its width today (Heine et al., 2013). Given that most cichlids today are freshwater fishes, marine dispersal over such long distances appears highly *improbable* and is often ruled out by advocates of Gondwanan vicariance (Sparks and Smith, 2005). However, there are reasons why oceanic dispersal of cichlids could nevertheless be *possible*:

First, several species of cichlid fishes have been shown to be capable to survive prolonged exposures to high salinity, including fully marine conditions. The most extreme of these may be *Oreochromis mossambicus* (Peters, 1852), which has been shown to survive in pure seawater (35‰) for as long as seven years in experiments (Myers, 1938; Nigrelli, 1940; Myers, 1949) and was observed breeding in hypersaline lakes at salinities up to 120‰ (Cyrus and Vivier, 2006; Vivier et al., 2010). Breeding populations of *Oreochromis mossambicus* are also known from several marine habitats. Examples of this include the central Pacific Fanning Atoll where the species was introduced in the 1950s and has since colonized various estuaries and the lagoon of the atoll (Lobel, 1980). The salinity of these estuaries and the lagoon vary but range up to 42.3‰ (Guinther, 1971). In addition, breeding populations of *Oreochromis mossambicus* are also known from coastal marine waters of California where it has co-occurred with *Coptodon zillii*

Gervais, 1848, since both species were introduced in 1973 (Knaggs, 1977).

Similar observations of salinity tolerance have been made for other cichlid species, including members of Etroplinae (e.g., *Etroplus maculatus* (Bloch, 1795) and *E. suratensis* (Bloch, 1790); Parvatheswararao, 1967; Chandrasekar et al., 2014) and Cichlinae (e.g., *Vieja maculicauda* (Regan, 1905) and *Mayaheros urophthalmus* (Günther, 1862); Oldfield, 2004) as well as further members of Pseudocrenilabrinae (e.g., *Oreochromis aureus* (Steindachner, 1864) and *O. niloticus*; Stickney, 1986; Avella et al., 1993; Nugon, 2003). To explain the presence of *Oreochromis aureus* in Israel, it is commonly assumed that the species "migrated through the Nile into the Israeli coastal rivers system" (Werner and Mokady, 2004), a passage that would have involved the crossing of tens or hundreds of kilometers of marine environment. In the Caribbean, the Neotropical cichlids *Vieja maculicauda* and *Mayaheros urophthalmus* "can regularly be found in the ocean" (Oldfield, 2004). Of the two species, *Mayaheros urophthalmus* has been observed spawning in pure seawater about 5 km off the coast of Belize, and *Vieja maculicauda* has been caught on the shores of Corn Island, about 50 km off the coast of Nicaragua (Conkel, 1993; Oldfield, 2004). Given that Corn Island is less than 5 km in diameter, has hardly any freshwater supplies, and cichlids are not commonly observed there, the continued presence of an endemic population is unlikely; instead, it is more probable that the observed individual dispersed from the coast of mainland Nicaragua. The occurrence of cichlids in marine habitats is also documented in the fossil record, as cichlid fossils were recently reported from marine sediments in Costa Rica (Lucas et al., 2017).

Examples of salinity tolerance in cichlids have been used before to argue for the possibility of oceanic dispersal (Murray, 2001a; Briggs, 2003), but were dismissed by Sparks and Smith (2005) who wrote that "the ability of one or even a handful of species to temporarily penetrate a few hundred meters into a marine environment does not imply a capability to cross hundreds or thousands of kilometers of open ocean." However (besides the fact that cichlids have been observed not only a few hundred meters, but up to 50 km into the marine environment), I assume that the perception of this incapability results from (i) a failure to recognize that only a single species is required for a transoceanic dispersal event, and (ii) a failure to fully appreciate the timescales at play. Of course, we would not expect that any of the above-named species would cross a marine distance of hundreds or thousands of kilometers before our eyes. But it should not come as a surprise that over timescales of tens of millions of years, events will happen that are more extreme than those that we observe during our lifetime. We also would not expect to observe a 10-km asteroid colliding with the Earth, wiping out 75% of all species, and yet exactly this type of event took place on the same timescales as those relevant for cichlid dispersal (Jablonski, 1994; Schulte et al., 2010). The reason why few of us are worried about asteroid impacts is that the per-year probabilities for such events are extremely low—on the order of one in 140 million (Chapman and Morrison, 1994; Harris, 2008). It is only over the long timescales of many millions of years that these probabilities become significant: Over 100 million years, the probability of an impact leading to a global catastrophe is around $1 - (1 - 1/140,000,000)^{100000000} = 0.51$. And similarly, transoceanic dispersal of cichlids over these timescales becomes probable even if we only assume that the per-year probability of oceanic dispersal is as low as one in 140 million. Thus, given that we have already observed marine dispersal of cichlids over distances of 5–50 km, can we confidently argue that the per-year probability of transoceanic dispersal (650–900 km) *must be lower than one in 140 million?* I don't think we can, which means that the possibility of transoceanic dispersal cannot be rejected a priori, and that we should therefore not disregard the molecular evidence at hand that, taken together, points to dispersal that probably took place some time between 75 and 60 Ma.

The above consideration does not yet take into account recent findings that further support the possibility of dispersal. One of these is that the marine family Pholidichthyidae, distributed in the Western Pacific, has recently been identified as the sister lineage of cichlids (Eytan et al., 2015). This sister-group relationship suggests that the common ancestor of all cichlids may also have been marine initially, and that ancestral species may still have been better adapted to the marine environment than most cichlids are today; this would be in line with the observation of high salinity tolerance in Etroplinae, the oldest of the four cichlid subfamilies (Parvatheswararao, 1967; Chandrasekar et al., 2014). Another recent finding supporting oceanic dispersal comes from an investigation of the parasite faunas of the different cichlid subfamilies, which showed that Indian and African cichlids share the same genus of monogenean endoparasites (*Enterogyrus*) but possess different ectoparasites (Pariselle et al., 2011; Vanhove et al., 2016). Since these ectoparasites are known to have a low tolerance to salinity variation, Vanhove et al. (2016) proposed that the different patterns for endo- and ectoparasites could be explained by prolonged exposure to high salinity during oceanic dispersal.

Finally, probabilities of dispersal have likely not remained constant over millions of years, but may have been promoted at certain times due to extreme external influences. As one such example, the influence of tsunamis on species dispersal has recently been forcefully demonstrated by the 2011 East Japan earthquake. The tsunami resulting from this earthquake reached a height of over 38 m on the coast of Japan, and the backflow washed millions of objects into the Pacific (Gewin, 2013). Six years later, as many as 289 coastal invertebrate and fish species had arrived on the shores of North America, "none of which were previously reported to have rafted transoceanically between continents" (Carlton et al., 2017). Of course, a large number of far more devastating tsunamis must have occurred over the past 100 million years, caused by earthquakes, landslides, or even meteorite impacts (Matsui et al., 2002). It is plausible to assume that if one of these megatsunamis would have hit the western shores of Africa, estuarine cichlids could have been washed far into the Atlantic, which might have led to long-distance dispersal.

Naturally, questions remain that cannot be answered by the dispersal hypothesis. For example, it has been noted that the sequence of the divergences of Malagasy, Indian, African, and Neotropical cichlids apparently matches the sequence of separations of the respective landmasses (Chakrabarty, 2004). While this coincidence would be expected under Gondwanan vicariance, its probability is one in six ($1/3 \times 1/2$) if transoceanic dispersal is assumed (or somewhat higher if we assume that the dispersal probability decreases gradually with increasing continental separation; Upchurch, 2008). Similarly, the dispersal hypothesis also does not explain why apparently each landmass was colonized only once (assuming that the ancestor of cichlids lived on Madagascar, and not counting Levantine or Iranian members of Pseudocrenilabrinae), whereas the Mozambique Channel with a width of only around 400 km should have been far easier to cross than the Atlantic and thus repeated crossings leading to sister-group relationships between African and Malagasy lineages might be expected (Sparks and Smith, 2005). Under the dispersal hypothesis, the best explanations for this are that either secondary colonizations were not successful due to competition from cichlids that had arrived earlier, or that due to chance alone, no landmass was colonized twice. Either way, these arguments against the dispersal hypothesis do not appear strong enough to fully exclude the possibility of transoceanic dispersal in cichlids. Thus, as argued above, the weight of the molecular evidence should be considered, which as a whole supports trans-Atlantic dispersal that probably took place at around 75–60 Ma.

Future directions

Owing to recent genome-sequencing efforts for various groups of cichlid fishes, we will soon be in the position to determine the timeline of cichlid diversification with unprecedented precision. Large nuclear datasets that include representatives of all cichlid tribes as well as outgroup taxa will enable us to overcome limitations of the previous studies, by reducing alignment saturation to a minimum while allowing the placement of fossil constraints directly on those branches to which the fossils were assigned taxonomically. However, to fully leverage the strength of these large molecular datasets, researchers using these data for divergence-time estimation should take care to avoid the repetition of previous mistakes: Importantly, fossil constraints used in these studies should be specified based on a quantitative model of diversification and fossilization, for example, using the approaches of Heath et al. (2014), Gavryushkina et al. (2017), or Matschiner et al. (2017). The phylogenetic placement of key cichlid fossils, such as *Mahengechromis* or the fossils from the Lumbrera Formation, should ideally be investigated further with more extensive morphological character matrices to corroborate or improve their current taxonomic assignment. Remaining uncertainty in the taxonomic affiliations of fossils should preferably be accounted for in the analysis, either by estimating their placement as part of the analysis (Ronquist et al., 2012a; Gavryushkina et al., 2017) or by allowing multiple alternative placements for one and the same fossil (Guindon, 2018). With the possible exception of fossils of uncertain taxonomic assignment, as many fossil constraints as possible should be included in the analysis to take maximum advantage of the valuable information provided by the fossil record. Like in all eleven Bayesian dating studies discussed in this review, a relaxed-clock model (Drummond et al., 2006; Lepage et al., 2007; Rannala and Yang, 2007) should be used to account for rate variation among the species included in the analysis, as the alternative use of the strict-clock model may lead to unreliable age estimates when the clock is violated (dos Reis et al., 2016). On the other hand, relaxed-clock models have also been criticized for being too uninformative, to the point that the resulting divergence-time estimates are often driven mostly by the specified prior densities (Brown and Smith, 2017). To address this issue at least partially, separate clock models can be applied to a partitioned dataset, which has been shown to improve the precision of divergence-time estimates (Zhu et al., 2015). Finally, future studies should ideally employ the multispecies coalescent model to account for the possibility of incomplete lineage sorting, which might further reduce potential bias in divergence-time estimates (Maddison, 1997; Ogilvie et al., 2017; Stange et al., 2018).

The computer programs BEAST 2 (Bouckaert et al., 2014) and RevBayes (Höhna et al., 2016; a successor to MrBayes; Ronquist et al., 2012b) lend themselves ideally to future studies of the timeline of cichlid diversification, because they co-estimate the phylogeny and the divergence times jointly through Bayesian inference, allow the specification of fossil constraints based on a model of diversification and fossilization (Gavryushkina et al., 2017; Matschiner et al., 2017), and implement a variety of clock models as well as the multispecies coalescent model (Drummond et al., 2006; Heled and Drummond, 2010). Another Bayesian program that co-estimates the phylogeny and the divergence times is PhyloBayes (Lartillot et al., 2009), a distinguishing feature of which is a mixture model for across-site variation in the substitution process. However, the use of fossil constraints in analyses with PhyloBayes is rather limited, and the multispecies coalescent model has not been implemented in the program. The drawback of all three "fully Bayesian" programs is their great computational demand, particularly when the datasets used for the analysis consist of genomewide sequences for several hundreds of taxa. Depending on the computational resources available to investigators, it may thus be more convenient to apply Bayesian tools that assume a fixed species tree and estimate only the branch lengths of the phylogeny. These tools include MCMCTree (a successor to multidivtime; Thorne et al., 1998) that is distributed as part of the PAML software package (Yang, 2007) as well as PhyTime, a dating method implemented in the PhyML package (Guindon, 2010; Guindon, 2012). Both programs are much faster than BEAST (Guindon, 2010; Battistuzzi et al., 2011), and PhyTime has the notable advantage of allowing for uncertainty in placement of fossils (Guindon, 2018). However, neither of the two programs implements a model of the fossilization process or the multispecies coalescent model.

With extremely large datasets or very limited computational resources, even the above programs assuming a fixed species tree may be too computationally demanding. In these cases, non-Bayesian tools may be useful, such as the RelTime method implemented in MEGA (Tamura et al., 2012). However, while RelTime has been shown to be extremely fast and can be applied to datasets containing thousands of taxa, a major limitation of the method is that age constraints can only be specified with hard boundaries. The reliability of age estimates produced by RelTime appears to be controversial, as Lozano-Fernandez et al. (2017) claimed that these are essentially based on a strict-clock model, whereas the software authors argue that the findings of Lozano-Fernandez et al. (2017) were due to a "lack of full equivalence between Bayesian and RelTime analysis conditions" (Battistuzzi et al., 2018). Thus, until the controversy has been resolved, it may be advisable to view results produced with RelTime with caution (Lozano-Fernandez et al., 2017) and to support these at least with Bayesian analyses of reduced datasets if the divergence-time estimates are used to draw conclusions about cichlid phylogeography.

Conclusion

Despite the partially contrasting results, molecular-clock studies have already provided valuable evidence toward a reliable timeline of cichlid diversification. In this review, I have discussed potential biases in the individual studies that may explain most, if not all, of the differences among the reported timelines. Overall, the molecular evidence supports a divergence of Pseudocrenilabrinae and Cichlinae subsequent to the breakup of the African and South American continents, probably around 75–60 Ma. This age estimate would imply that cichlid fishes crossed the Atlantic when the ocean was already at least 650 km wide, and that they probably also crossed the Mozambique Channel and the Indian Ocean after the separations of Madagascar and India. Given that most cichlid fishes are limited to freshwater habitats, marine dispersal over such wide distances might seem improbable; however, the high salinity tolerance of several cichlid species, together with observations of cichlids in the marine environment, suggests that the possibility of oceanic dispersal of cichlids should not be excluded and that the molecular evidence should therefore be given full consideration. Due to recent progress in genome-sequencing of cichlid fishes, future studies will soon be able to shed more light on the divergence times of cichlids with far larger molecular datasets than previously available. If these studies avoid the pitfalls of previous studies as discussed in this review, it may be hoped that a robust and precise timeline of cichlid diversification will soon be available to allow further insights into the mechanisms behind their spectacular evolutionary success.

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