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Contribution to the molecular phylogenetic analysis of extant holocephalan fishes (Holocephali, Chimaeriformes)

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Abstract Much attention has been paid to the molecular phylogeny of holocephalan fishes during recent years, but sampling was very low and not all genera were examined. This study offers an extended sampling of species from all known genera to clarify their phylogeny and to provide an estimate of the time of origin of extant holocephalan taxa. Three mitochondrial genes (cytochrome b, 12S rRNA, and 16S rRNA) were sequenced and analysed using a variety of phylogenetic methods (Bayes, maximum likelihood, and maximum parsimony). Callorhinchidae diverged from Rhinochimaeridae and Chimaeridae about 187 Ma ago. Chimaeridae and Rhinochimaeridae diverged from each other about 159 Ma ago. Within Rhinochimaeridae, *Neoharriotta* is the sister genus to the closely related *Harriotta* and *Rhinochimaera*. Eight species of the family Chimaeridae, belonging to the genera *Hydrolagus* and *Chimaera*, were examined. They probably had a common ancestor about 107 Ma ago and appear paraphyletic. These results indicate that the traditional morphological generic definition of the families Rhinochimaeridae and Chimaeridae has to be reinvestigated.

Keywords Chondrichthyes · Chimaeridae · Molecular phylogeny · Evolution · *Neoharriotta pinnata* · Node time estimation

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Introduction

The holocephalan or chimaeroid fishes are an enigmatic, mostly deep-water group of chondrichthyans - "cartilaginous fishes". They have a worldwide distribution except in Arctic and Antarctic waters, although some species are restricted to seas of the Southern Hemisphere (Didier 2004).

Extant chimaeras are slow swimming, bathydemersal fishes. They show many features considered characteristic for this lifestyle, for example their elongate body form and a more or less reduced caudalis, as well as huge enlarged pectoral fins (Fig. 1). Adults of most species grow to a total length of about 50 cm, but some species can reach a length of more than 1 m. Nevertheless, little is actually known of the biology of this group, and most information essentially comes from two species, *Hydrolagus colliei* (Lay & Bennett, 1839) and *Callorhinchus milii* Bory de St. Vincent, 1823.

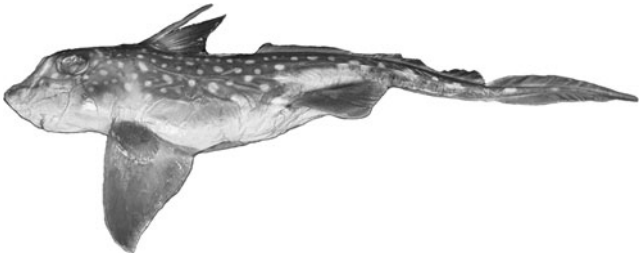

Fig. 1 *Hydrolagus colliei* from the east Pacific

These two species are more easily accessible than other members of the group as they live in waters of moderate depth off the western coast of North America and around New Zealand, respectively (e.g. Didier and Rosenberger 2002).

Comprising six genera and about 40 described species, the three extant holocephalan families Chimaeridae, Rhinochimaeridae, and Callorhinchidae, belong to a clade of fishes that dates back to the Paleozoic (e.g. Didier 1995; Stahl 1999). The modern chimaeriform fishes are thus survivors of a previously more successful and diverse group (Stahl 1999; Helfman et al. 2009). They had their supposedly greatest diversity during the Carboniferous period (350-300 Ma), but there are still large gaps in the fossil record (e.g. Patterson 1965) and most lineages became extinct at the end of the Permian (250 Ma). Moreover, the relationships and crucial apomorphic characters of groups of fossils and recent holocephalans are often questionable and still much debated (Patterson 1965; Stahl 1999).

During the nineteenth and twentieth centuries, research was carried out on the morphology and phylogeny of the Chimaeriformes (e.g. Leydig 1851; Hubrecht 1877; Cole 1896a, b, c; Dean 1906, 1909; Allis 1917, 1926; De Beer and Moy-Thomas 1935; Patterson 1965; Stahl 1967, 1999; Maisey 1986; Didier 1995; Lund and Grogan 1997; Grogan et al. 1999; Liu 2001). In most cases, the chimaeroid fishes were seen as closely related to the Elasmobranchii and forming the monophyletic Chondrichthyes. Several authors (e.g. Schaeffer and Williams 1977; Schaeffer 1981; Zangerl 1981; Maisey 1986) described morphological features that they regarded as synapomorphic for Chondrichthyes. By contrast, the close relationship of Chimaeriformes to the extinct plagiostome group Ptyctodontiformes – and accordingly some common ancestry among placoderms or Arthrodira – has been proposed for a long time (e.g. Didier 1987; Holmgren 1942; Licht 2008; Ørvig 1960, 1962).

Notwithstanding their still unresolved sister-group relationships, the intra-relationships of the modern holocephalan fishes have also not yet been completely resolved. Morphological analyses have offered little insight into the systematics of the extant representatives of this old group of fishes and relationships among the various genera are still unclear (e.g. Didier 1995; Maisey 1986; Stahl 1999). Lively interest in the molecular analysis of holocephalan systematics by evolutionary biologists has arisen in recent years (e.g. Heinicke et al. 2009; Inoue et al. 2010; Ward et al. 2008). Especially the origin, systematics, and "barcoding" of extant chimaeroids have been the focus of these recent publications. These studies, however, did not examine all genera, nor was the number of included species sufficient for a comprehensive reconstruction of holocephalan phylogeny. There are still open questions concerning relationships, as the results of earlier studies are, in some cases, still inconsistent. For example, Ward et al. (2008) published a neighbour-joining tree based on the Cox1 gene with Australian holocephalans of the genera *Chimaera* and *Hydrolagus* (both Chimaeridae). In this particular tree one species of *Hydrolagus* is nested within a clade of all studied species of *Chimaera*. By contrast, Inoue et al. (2010) found that these two genera were monophyletic, based on the whole mitochondrial genome. In further studies holocephalan fishes were mostly used as an outgroup for other molecular studies or only a few species were examined (e.g. Arnason et al. 2001; Douady et al. 2003; Le et al. 1993; Rasmussen and Arnason 1999; Ward et al. 2005).

The main aim of this study is an extended molecular phylogeny including for the first time all recognised genera with a larger species sampling – based for the first time on all genera from former studies – to clarify the intra-relationships of extant holocephalans. In addition, we aim to provide a time estimate of holocephalan origins in order to compare our results with former studies and give clues about future further studies on their life history.

Materials and methods

Taxon sampling

The sequenced specimens and species used in this study are listed in Table 1. Samples were pieces of muscle tissue, which were fixed in 75% ethanol. We expanded the species sampling by Inoue et al. (2010) by the inclusion of six further species: *Neoharriotta pinnata* (Schnakenbeck, 1929), *Chimaera phantasma* Jordan & Snyder, 1900, *Hydrolagus colliei* (Lay & Bennett, 1839), *Hydrolagus africanus* (Gilchrist, 1922), *Hydrolagus* cf. *alberti* Bigelow & Schroeder, 1951, and *Hydrolagus novaezealandiae* (Fowler, 1910). We also used Genbank sequences of *Squalus acanthias* Linnaeus, 1758 (NC_002012), *Scyliorhinus canicula* (Linnaeus, 1758) (NC_001950), *Mustelus manazo* Bleeker, 1854 (NC_000890), *Amblyraja radiata* (Donovan, 1808) (NC_000893), *Okamejei kenojei* (Müller & Henle, 1841) (NC_007173), *Rhinochimaera pacifica* (Mitsukurii, 1895) (NC_014293), *Callorhinchus callorhynchus* Linnaeus, 1758

Table 1 Specimens sequenced in this study with localities and GenBank accession numbers

Collection number	Species	Region	Acc. No. cytb	Acc. No. 125 rRna	Acc. No. 165 rRna
MTD 3996 (Dresden)	*Callorhinchus milii*	South Australia	JN_703276	JF_773764	JN_703267
ZSM 32809 (Munich)	*Callorhinchus capensis*	Angola	JN_703277	JF_773765	JN_703268
ZSM 40419 (Munich)	*Harriotta raleighana*	New Zealand	JN_703272	JF_773760	JN_703263
A 230 (Chicago)	*Neoharriotta pinnata*	Angola	JN_703273	JF_773761	JN_703264
ZSM 40415 (Munich)	*Hydrolagus novaezealandiae*	New Zealand	JN_703280		JN_703271
A 461 (Chicago)	*Hydrolagus africanus*	Angola	JN_703278	JN_703261	JN_703269
B 367 (Munich)	*Hydrolagus* cf. *alberti*	Angola	JN_703279	JN_703262	JN_703270
ZSM-tissue-collection-P. CH_0254 (Munich)	*Chimaera phantasma*	Japan	JN_703274	JF_773762	JN_703265
? (Frankfurt)	*Chimaera monstrosa*	Western Mediterranean	JN_703275	JF_773763	JN_703266
Species from genbank and Inoue et al. 2010	Acc. No.				
Rhinochimaera pacifica	NC_014293				
Callorhinchus callorhynchus	NC_014281				
Hydrolagus lemures	NC_014290				
Hydrolagus colliei	AY_147899.1; EF_119279.2; AY_973060.1				
Chimaera fulva	NC_014266				
Petromyzon marinus	NC_001626.1				
Squalus acanthias	NC_002012				
Scyliorhinus canicula	NC_001950				
Mustelus manazo	NC_000890				
Amblyraja radiata	NC_000893				
Okamejei kenojei	NC_007173				

(NC_014281), *Hydrolagus lemures* Whiteley, 1939 (NC_014290), *Hydrolagus colliei* (Lay & Bennett, 1839) (AY_147899.1; EF_119279.2; AY_973060.1), and *Chimaera fulva* Didier, Last & White, 2008 (NC_014288), and as the outgroup *Petromyzon marinus* Linnaeus, 1758 (NC_001626.1).

Molecular methods

We used partial sequences of the mitochondrial genes 12S rRNA, 16S rRNA, and cytochrome b (cyt*b*) in order to achieve the best match with the sequence data from previous studies (Inoue et al. 2010).

In order to avoid any kind of contamination, DNA extraction and PCR from two separate muscle tissue samples of each specimen were carried out in a separate clean laboratory, and each step of the analysis (sampling, extraction, and PCR) was done under separate sterile benches. DNA was extracted using ANALYTIK JENA innuPREP DNA Mini Kit according to the manufacturer's instructions with small modifications: an elution volume of 75 μl instead of 200 μl and digestion overnight.

Each PCR was performed in a 25-μl volume using 1-2 μl eluted DNA as a template (4-6 μl of eluted aDNA), 3 μl of PCR buffer (including Mg), and 1 μl of each forward and reverse primer, 1 μl DNTp and 0.2 μl Taq-polymerase (filled up to a reaction volume of 25 μl).

From fresh tissue samples in general the entire partial fragments for all three markers described below, could be amplified in a single PCR. However, due to degradation of DNA, over time older samples from museum collections had to be amplified in two smaller fragments that were subsequently then concatenated with regions of overlap of 57 to 78 base pairs. Cytochrome b: A 655-bp-long fragment of the cytochrome *b* gene was amplified in a double-stranded PCR using the newly designed primer combinations Chimaera-Cyt*b*F1 (5'- CTM CGA AAA WCC CAC CC -3') and Chimaera-Cyt*b*R1 (5'- ACG AAG GCT GTT ATT ATT AC -3') for the first part, and Chimaera-Cyt*b*F2 (5'- GCY TCC TTA TTY TTT ATT TGC C -3') and Chimaera-Cyt*b*R2 (5'- ATA AAG TAG GGR TGG AAG G -3') for the second part. Amplification of the first Cyt*b* fragment was carried out under the following PCR conditions: 94°C for 2 min and 35 cycles of 94°C for 45 s, 54° C for 1 min and 72°C for 1 min. The second Cyt*b* fragment

was amplified under slightly modified conditions: 94°C for 2 min and 35 cycles of 94°C for 45 s, 58°C for 1 min and 72° C for 1 min.

A partial fragment of approximately 547 bp (varying with the number of indels) of the 16S ribosomal RNA gene was amplified using the newly designed primer combinations Chimaera-16SF1 (5'- CGC CTG CCC TGT GAC -3') with Chimaera-16SR1 (5'- ACC CCG TGG TTG CCC -3') and Chimaera-16SF2 (5'- CGA GAA GAC CCT ATG GAG C -3') with Chimaera-16SR2 (5'- AAC AAA CGA ACC CTT AAT AGC G -3'). PCR conditions for amplification of the first 16S rRNA fragment were: 5 min and 35 cycles of 95° C for 30 s, 52°C for 45 s and 72° C for 1 min. The second 16S rRNA fragment was amplified under the following conditions: 95°C for 5 min and 30 cycles of 95°C for 30 s, 54°C for 45 s and 72°C for 1 min.

A partial fragment of approximately 763 bp (varying with the number of indels) of the 12S ribosomal RNA gene was amplified using the newly designed primer combinations Chimaera-12Sfor (5'- TTA CAC ATG CAA GTT TCC GC -3') and Chimaera-12Srev (5'- CTY AGA GCC ATT TTC AGA TT -3'). Amplification of the 12S rRNA fragment was carried out under the following conditions: 95° C for 5 min and 35 cycles of 95° C for 30 s, 54° C for 45 s and 72° C for 1 min.

PCR products were purified using ExoSap-IT (GE Healthcare; adding 0.1 μl ExoSap-IT solution in 4 μl H_2O dest. to each sample; cycling program: 37°C for 30 min and 94°C for 15 min) and sequenced in both directions on an ABI3700 DNA sequencer. Sequencing of the PCR products was performed with BigDye™ v. 3.0 and v. 3.1 Dye Terminator Cycle Sequencing Kits (Applied Biosystems) according to the manufacturer's instructions. Reactions were electrophoresed with the ABI 377 automatic sequencer.

The sequences were aligned by ClustalW using MEGA 3.1 (Kumar et al. 2004) and slightly adjusted by eye, where needed. All sequences used for the analysis were deposited at GenBank (Table 1).

Data analysis

The appropriate substitution model for each of the three sequence data sets was estimated for each of the three genes analysed using MrMODELTEST (Nylander 2004). According to the Akaike Information Criterion the best fit model was the GTR model for all mitochondrial markers with the following likelihood settings for the cytb data set (the same settings were estimated for likelihood and Bayesian analysis): GTR+I+G, empirical base frequencies: πA=0.2928, πC=0.3021, πG=0.1003, πT=0.3048; proportion of invariable sites I=0.4314; gamma shape parameter α=1.0252; rate matrix: R(a)[A-C]=1.6174, R(b)[A-G]=10.4929, R(c)[A-T]=1.3428, R(d)[C-G]=1.3439, R(e)[C-T]=10.4475, R(f)[G-T]=1.0000.

Model settings for analysis of the 12S rRNA data set were estimated as follows: GTR+G, empirical base frequencies: πA=0.3657, πC=0.2029, πG=0.1731, πT=0.2582; proportion of invariable sites I=0; gamma shape parameter α=0.3555; rate matrix: R(a)[A-C]=7.6600, R(b)[A-G]=24.6704, R(c)[A-T]=11.9733, R(d)[C-G]=0.2729, R(e)[C-T]=63.5945, R(f)[G-T]=1.0000.

Model settings for analysis of the 16S rRNA data set were estimated as follows: GTR+I+G, empirical base frequencies: πA=0.3288, πC=0.2030, πG=0.1803, πT=0.2879; proportion of invariable sites I=0.4613; gamma shape parameter α=0.6865; rate matrix: R(a)[A-C]=5.4461, R(b)[A-G]=16.7451, R(c)[A-T]=9.3196, R(d)[C-G]=3.3664, R(e)[C-T]=33.1168, R(f)[G-T]=1.0000.

Phylogenetic trees were reconstructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) implemented in the programs PAUP* 4.0b10 (Swofford 2002), raxML 7.2.8 (Stamatakis 2006; Silvestro and Michalak 2011), and MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), respectively. MP and ML analyses were performed in a heuristic search with the TBR branch swapping option. Clade support in ML analysis was estimated with 10^6 rearrangements and gaps coded as a fifth character state. Clade support in MP analysis was estimated by 1,000 bootstrap replicates (Felsenstein 1985) in a fast heuristic search with all characters unordered and equally weighted, and gaps treated as a fifth character state. Bayesian analysis was performed using the Metropolis-coupled Markov chain Monte Carlo algorithm with two parallel runs, each with one cold and three heated chains. The heating parameter λ was set to 0.1 to obtain convergence. The chains ran for 10^6 generations with every 100th generation sampled (burn-in = 5,000). The remaining trees were used for generating a 50% majority rule consensus tree.

For reconstruction of a mitochondrial total evidence tree using Bayesian inference of phylogeny, we partitioned the concatenated sequence data by gene and codon into five different partitions corresponding to (1) the two RNA markers and (2) the cytochrome-b fragment partitioned by codon. We applied the best fit model to each partition and allowed the overall rate to vary between partitions by setting the priors<ratepr=variable>and model parameters such as gamma shape, proportion of invariable sites, etc., unlinked across partitions, so that for each partition a separate set of parameters was estimated. We applied the same partition by gene and codon to the data set in the raxML analysis. Applying the GTR+G+I model across all four partitions, we obtained for the best scoring ML tree thorough bootstrap support using 1,000 replicates.

Because recent studies showed that consideration of a rRNA secondary structure can significantly improve both

sequence alignment and phylogenetic reconstructions (Letsch et al. 2010; Letsch and Kjer 2011), we carried out two further independent runs with MrBayes. For prediction of rRNA secondary structure, we used the RNAstructure software package (Reuter and Mathews 2010) and compared the structures with our alignment to identify loops and erratic aligned regions. Based on these results, the following 13 regions were excluded from the analysis of the concatenated alignment: 12S rRNA: 4-9, 34-43, 62, 102-107, 136-141, 197-203, 222-263, 281-294, 324-336, 347-352, 393-425, 612-617, 626, 679-698, 708-716, 737-745; 16S rRNA: 962-1033, 1082-1095, and 1155-1162. Notably, we kept unambiguously aligned conservative regions that were not clearly identified as stems or loops in the structures to avoid exclusion of informative positions.

Thus, the subsequent reconstructions should have been based on conservative regions of our rRNA markers (presumably stem regions) and should provide a robust phylogenetic hypothesis. Again the corrected alignment was partitioned by gene and codon including all three codon positions of the cytochrome-b partition under the NY98 model in the first run with MrBayes (two parallel runs, each with one cold and three heated chains, Markov chain length = 7,500,000 generations, sampling frequency = 1.000 and burn-in = 3.500) – the second run differed only by the exclusion of the third codon position from the analysis.

Based on the concatenated data set of the three mitochondrial DNA fragments, split ages for the phylogeny were estimated by a relaxed molecular clock as implemented in BEAST 1.5.4 (Drummond and Rambaut 2007). We applied a fossil-based molecular dating approach largely based on the calibration by Inoue et al. (2010). For calibration of node ages we referred to *Stensioella heintzi* Broili, 1933, as the oldest holocephalan (~410 Ma; node 1 in Tables 2, 3; node 9 in Inoue et al. 2010), *Eomanodon simmsi* Ward & Duffin, 1989, as a potential ancestor of Callorhinchidae and Chimaeroidea (according Duffin 2001, ~183 Ma, node 6 in Tables 2, 3; node 16 in Inoue et al. 2010), *Elasmodectes* from the Kimmeridgian as the oldest known Rhinochimaeridae and Chimaeridae (according Duffin 2001, ~156 Ma, node 9 in Tables 2, 3; node 16 in Inoue et al. 2010) and one further fossil age for the lineage split among Batoidea and Selachii (defined by the earliest fossil records of Synechodontiformes, Klug 2010, ~250-295 Ma, node 2 in Tables 2, 3; node 19 in Inoue et al. 2010).

We applied a lognormal tmrca prior distribution, and all fossil ages were used as a hard minimum age constraint (lognormal offset) to the respective nodes. In order to create a lognormal distribution that matches tmrca priors of calibration nodes in real space (along the stratigraphic scale), the lognormal mean and standard deviation for each fixed node were set according to a soft maximum constraint using the next older fossil age on the same clade. In fossil calibrations, the soft maximum constraint can be used as an upper limit of a lognormal prior distribution in order to decrease the probability density extending back in time from the minimum constraint (review in Benton et al. 2009). A rough inference of a soft maximum constraint can be done by using the age of the oldest fossil belonging to the nearest relative, sister group, or setting as a limit of "the next oldest fossil horizon that lacks relevant fossils" (Benton and Donoghue 2007; note that this approach was later on challenged by Benton et al. 2009). For instance, the split among holocephalans and elasmobranchs was assigned a hard minimum constraint according to the age of the oldest known holocephalan fossil *Stensioella heintzi* from the lower Devonian, Pragian (lorgnormal offset=410; see above). Also from the lower Devonian (lowermost Lochkovian) the oldest chondrichthyan shark fossils are known (Botella et al. 2009). Thus, the Silurian-Devonian boundary was set as the soft maximum for the holocephalan-elasmobranch split by adjusting the lognormal prior distribution accordingly (mean=1.0, SD=0.5, ~416 Ma, node 1 in Table 3). For fossil evidence and tmrca prior distributions applied to the remaining three nodes; see Table 3.

The length of the MCMC chain was set to 30 million generations, and log parameters were sampled every 1,000th generation. We chose a lognormal relaxed clock model (Drummond et al. 2006) with tree prior set to speciation (yule process) and the "auto optimize" option was activated in order

Table 2 Divergence time estimates in comparison with former molecular studies (in Ma)

Node	This study	95% HPD	Inoue et al. 2010	Heinicke et al. 2009
1	413	[411-417]	421	471
2	264	[252-284]	281	393
3	164	[131-200]	225	350
4	110	[79-142]	169	227
5	71	[48-98]	66	—
6	187	[183-193]	167	220
7	11	[7-16]	6	—
8	8	[6-12]	3.7	—
9	159	[156-164]	122	107
10	109	[89-131]	—	—
11	58	[40-77]	47	—
12	107	[88-128]	—	—
13	98	[79-117]	—	—
14	81	[61-101]	—	—
15	1, 1	[0.1-2.4]	—	—
16	50	[34-67]	—	—
17	15	[8-23]	—	—
18	22	[13-32]	—	—

Table 3 Fossils used for calibration of four nodes of the chondrichthyan phylogeny; molecular dating with BEAST, lognormal tmrc prior distribution (lognormal offset, mean and standard deviation given for all nodes); the beginning of the next older stratum lacking the respective fossils was chosen as a soft maximum age for all nodes according to Benton et al. (2009)

	Min age	Max age	offset	mean	sd
Node 1	*Stensioella*, oldest holocephalan, 410 Ma, Pragium, (Oldest chondrichthyan shark tooth, 415 Ma)	Beginning of Lochkovion, 416 Ma	410	1	0.5
Node 2	215 End of Permian	oldest Neoselachian fossil, 295 Ma, Early Permian	251	2.5	0.75
Node 6	Oldest Callorhinchidae fossils, *Eomanodon*, *Brachymylus*, 183 Ma, early Pliensbachian	Beginning of Sinemurian, 196.5 Ma	183	1.25	0.75
Node 9	Oldest Rhinochimaerid, *Elasmodectes*, Kimmeridgian |155.7 Ma	Beginning of Oxfordian, 161 Ma	156	1	0.25

to adjust the tuning parameters automatically. Input sequence data were manually partitioned according to the different gene fragments in the XML file generated with BEAUTi and the GTR+I+G model, respectively, and were *a priori* assigned to each partition according to the estimates with MRMODELTEST. A linearised consensus tree including posterior probabilities was inferred from the tree output files from the third approach (concatenated sequence data sets) using TreeAnnotator v1.4.8 (as implemented in the BEAST package) with the burn-in parameter set to 9,000. Confidence intervals (CI 95%) for time estimates of lineage splits and mean substitution rates were inferred from the log output files using TRACER software (Rambaut and Drummond 2007).

Results

Phylogenetic relationships

The 1,973-bp-long alignment of concatenated mitochondrial sequences included 931 constant characters, 776 parsimony-informative characters, and 266 singletons (*Petromyzon* included). The Bayesian total evidence tree is shown in Fig. 2 with bootstrap support from the MP and ML analyses added to the respective nodes. The topologies resulting from all three reconstruction methods (MP, ML, and Bayes regardless of consideration of codon partition or secondary rRNA structure) are largely identical except for four nodes that are marked in the total evidence tree (and commented on in the following). Consideration of the rRNA secondary structure had no effect on the topology of the Bayesian tree reconstructions. When the third codon position was excluded from the data set in run 4 (consideration of rRNA structure, partition by gene and codon, exclusion of third position), only two nodes resulted in a different grouping (see below, nodes A and B).

The 19 taxa used in this analysis form two main clades (Fig. 2). The first clade comprises the elasmobranch fishes (sharks and batoids). The second clade is formed by the holocephalan fishes. Both clades received maximum support from Bayesian inference of phylogeny and strong support from the ML analysis.

Within the holocephalans, all three families (Callorhinchidae, Rhinochimaeridae, and Chimaeridae) are recovered as monophyletic with strong support for each family from all analyses. Within Callorhinchidae a sister group relationship between *C. callorhynchus* and *C. milii* received moderate support from raxML analyses and from Bayesian inference of phylogeny when the third codon position of cytochrome b was excluded (node A, Fig. 2). Thus, within-clade relationships of *Callorhinchus* remained poorly resolved.

Rhinochimaeridae and Chimaeridae cluster together as sister groups opposed to Callorhinchidae, and this topology is reflected by all analyses. This sister group received strong support from all analyses (regardless of consideration of rRNA structure or partitioning by codon). Within Rhinochimaeridae *Rhinochimaera pacifica* turned out to be the closest relative of *Harriotta raleighana* Goode & Bean, 1895, with strong support from all reconstructions. We analysed eight species of Chimaeridae, five *Hydrolagus* and three *Chimaera*, and found that neither of these two genera is supported as a monophyletic clade. *Hydrolagus lemures* and *Chimaera phantasma* are sister taxa in all analyses with strong support, and also a sister group relationship of *Hydrolagus novaezealandiae* and *Chimaera monstrosa* Linnaeus, 1758 (Fig. 2), was strongly supported by all analyses except when the third codon position was excluded from Bayesian analysis, run 4 (node B, Fig. 2). Exclusion of the third codon position resulted in a poorly supported sister group relationship of *H. novaezelandiae* and *C. fulva* (not shown). However, all three species in question formed a monophyletic group with maximum support from all analyses.

The position of *Hydrolagus colliei* was equivocal among different phylogenetic reconstructions (Fig. 2): This species was sister of a clade comprising *H. africanus* and *H.* cf. *alberti* in all Bayesian trees (posterior

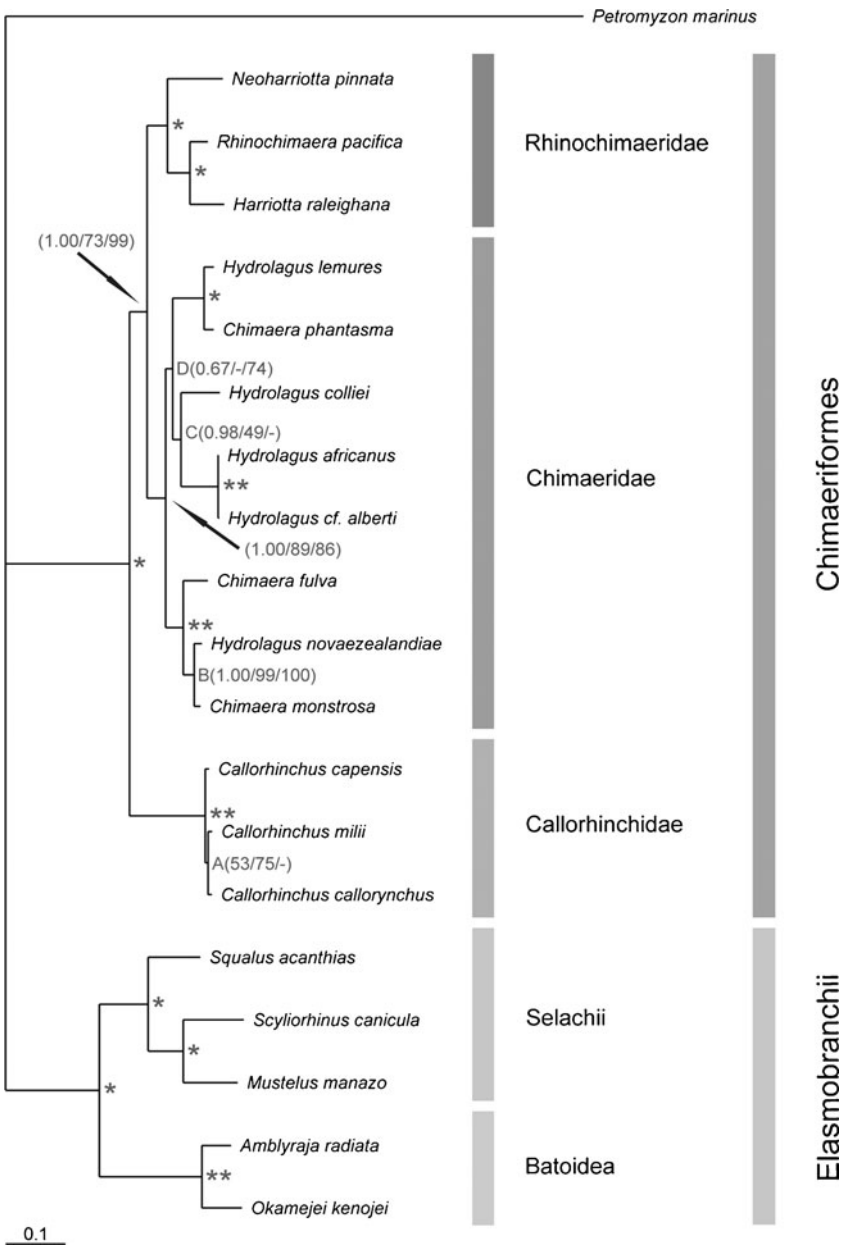

Fig. 2 Molecular phylogeny of the Chimaeriformes; Bayesian inference of phylogeny based on 1,793-bp concatenated sequences of three mitochondrial genes (cytb, 12S rRNA, 16S rRNA) MCMC 7,500,000 generations; node support given for Bayesian rRNA by gene and codon 1-3/ raxML bootstrap/MP bootstrap. (Two stars = maximum support all (1.00/100%); one star = strong support (BI=1.00/ raxML and MP≥95%). Treebase acc. no.: http://purl.org/phylo/treebase/phylows/study/TB2:S12051

support only slightly decreased when the third codon position was excluded) and in the raxML tree, however with poor support from thorough bootstrap analysis. The deep splits within Chimaeridae were poorly resolved regardless of reconstruction method. Most analyses supported a sister group relationship of a clade comprising *H. lemures* and *C. phantasma* with a second clade uniting *H. colliei*, *H. africanus*, and *H.* cf. *alberti* (node D, poor support from posterior probabilities, moderate support from MP). RaxML analyses and Bayesian inference of phylogeny under exclusion of the third codon position showed the *H. lemures*/*C. phantasma* clade as basal to the remaining chimaerid species, however with poor support for the crown group.

We recovered essentially the same results for the relationships between the species, or representatives of the three elasmobranch groups, like Inoue et al. (2010). Two main clades can be recognised within these fishes. The first is formed by the batoids and the second by sharks. Within the sharks the Galeomorphii and Squalea are separated into two branches. All subclades within the elasmobranchs received strong support (Fig. 2).

Divergence time estimates

The topology resulting from BEAST analysis was identical to those resulting from runs 1, 2, and 3 with MrBayes except for the poorly supported node D. The BEAST topology of

the chimaerid clade was identical to those calculated using raxML with *H. lemures*/*C. phantasma* clade representing the basal clade (Fig. 2, node D, posterior support for the crown clade was 0.75).

According to our estimates, the extant holocephalans diverged from a common ancestor with the elasmobranch fishes about 413 Ma before the present back in the Devonian. This separation has a 95% confidence interval of 411-417 Ma. The Callorhinchidae are the most basal family within the Chimaeriformes and split off from the holocephalan clade in the Jurassic about 187 Ma (183-193 Ma) ago (Table 2). The three species of Callorhinchidae are very young compared to most other holocephalan species pairs. They separated from each other relatively recently about 11 Ma ago during Mid Miocene times. The divergence between Rhinochimaeridae and Chimaeridae was estimated at about 159 Ma ago (156-164 Ma). The genus *Neoharriotta* diverged from the other two genera about 109 Ma ago (89-131 Ma), and the genera *Harriotta* and *Rhinochimaera* diverged 58 Ma (40-77 Ma) before present. All Chimaeridae were dated back to a common ancestor about 107 Ma ago with a 95% credibility interval of 88-128 Ma. Time estimates for lineage splits between chimaeroid species as well as elasmobranch fishes are given in Table 2 and Fig. 3.

Discussion

Molecular phylogenetic relationships of extant holocephalan fishes

All three holocephalan families (Callorhinchidae, Rhinochimaeridae, and Chimaeridae) are recognised in our study and recovered as monophyletic with strong support. These three families of extant holocephalan, or chimaeriform, fishes are also clearly confirmed by other morphological and molecular studies (e.g. Didier 1995; Heinicke et al. 2009; Inoue et al. 2010; Stahl 1999).

The Callorhinchidae is considered the most basal family within the holocephalans (e.g. Didier 1995; Heinicke et al. 2009; Inoue et al. 2010; Stahl 1999). This family consists of one genus with three species, *Callorhinchus capensis* Duméril, 1865, *C. milii*, and *C. callorhynchus*. All three species are hard to distinguish anatomically and are normally separated by their geographic distribution range alone (Didier 2004). Our results show that *C. capensis* is the sister species to the other two species, but the species relationships within the Callorhinchidae are not well supported (see above). In the study of Inoue et al. 2010, *C. callorhynchus* is the sister species of the other two species. Similar to our study relationships within this family were not well supported (ML 88%).

The Rhinochimaeridae and Chimaeridae are confirmed as a single clade, opposed to the Callorhinchidae. In two other recent molecular studies this sister group relationship is also supported (Heinicke et al. 2009; Inoue et al. 2010). In another study (Douady et al. 2003) it was concluded that Chimaeridae is paraphyletic, because a rhinochimaerid was embedded in the branchings among the chimaeroid taxa. Inoue et al. (2010) re-examined and blasted the same sequence, and were able to demonstrate that *Rhinochimaera pacifica* does indeed have a high sequence similarity with *Chimaera monstrosa*. Our results, however, confirm that the Chimaridae are monophyletic. This is supported by the fact that Rhinochimaeridae and Chimaeridae are easily separable on morphological grounds, as Didier (1995) demonstrated in her extensive anatomical study.

Rhinochimaeridae involve three genera (*Rhinochimaera*, *Harriotta*, and *Neoharriotta*), but only *Rhinochimaera* and *Harriotta* were used in recent molecular studies. Didier (1995) divided the three genera into two subfamilies based on their morphology. The first one, Rhinochimaerinae, erected by Didier (1995), comprises the genus *Rhinochimaera* alone. The second one, Harriottinae, was erected by Gill (1898) and contains the genera *Harriotta* and *Neoharriotta*. Rhinochimaerinae is distinguished from Harriottinae by e.g. smooth, thin tooth plates, which lack hypermineralized tissue; an elongate ethmoid region of the neurocranium; small orbits; presence of the retractor mesioventralis pectoralis muscle (Didier 1995). It is noteworthy that Harriottinae were not supported by any synapomorphies (Didier 2004).

The results of this study for all three genera show instead that *Rhinochimaera* is closely related to *Harriotta* (Fig. 2). *Neoharriotta* is more basal and the closest relative of the former two genera. These internal phylogenetic relationships within Rhinochimaeridae were strongly supported in the mitochondrial total evidence tree, and the subfamilies proposed by Gill (1898) and Didier (1995) cannot be supported by our molecular analysis (Fig. 2).

Chimaeridae comprise the genera *Hydrolagus* and *Chimaera*. They are normally distinguished by the presence (*Chimaera*) or absence (*Hydrolagus*) of an anal fin (e.g. Didier 2004). Inoue et al. (2010) confirmed that these two genera are monophyletic, although Ward et al. (2008) showed in their K2P distanced neighbour-joining tree based on Cox1 that *Hydrolagus* cf. *barbouri* is nested within the genus *Chimaera* and most closely related to *Chimaera panthera*. This was the first indication that the two supposed genera could be paraphyletic. In our study we analysed eight species, five *Hydrolagus* and three *Chimaera*, and the result is that neither of these two genera emerges as a monophyletic taxon. A possible multiple loss of the anal fin within the Chimaeridae

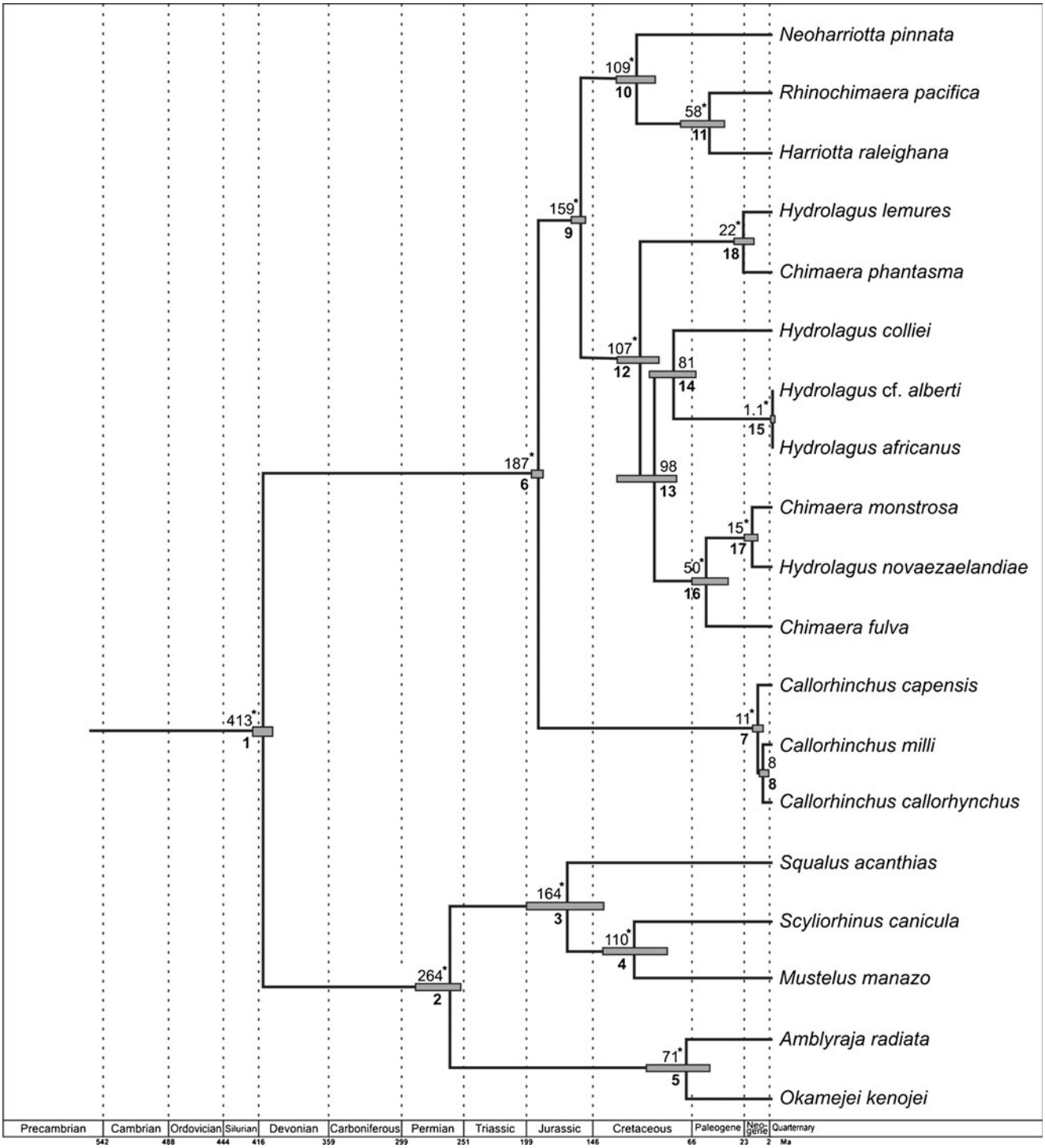

Fig. 3 Relaxed molecular clock estimates for ages of extant holocephalans; Bayesian inference of phylogeny (using BEAST 1.4.8) with three partitions corresponding to three mitochondrial DNA fragments, 10,000,000 generations, relaxed lognormal clock, tree prior = yule process (speciation); *horizontal bars* indicate credible intervals (95%) of the divergence time estimates. Mean age estimates for each node above *horizontal bars* and node numbers according to Table 2 below them. An asterisk marks clade support of 1.00 Bayesian posterior probabilities. Probabilities lower than 100% are: node 8 (71%), node 13 (75%), node 14 (97%)

is conceivable. A morphological study of the cranium between *Hydrolagus* and *Chimaera* showed that the differences between *Hydrolagus colliei* and *Chimaera monstrosa* are quite modest and more at a species rather than a genus level (Licht

2008; Licht and Bartsch 2009). Further studies concerning the morphology of chimaeroids are in progress.

Divergence time estimates and origin of extant holocephalan fishes

Although there are some differences between our molecular dating approach and those applied in former studies, the mean age estimates and confidence intervals for lineage splits within holocephalan fishes are highly congruent. While our phylogeny is very much focussed on Chimaeriformes and only four nodes were used for calibration (including one basal node as a soft maximum), former studies by Heinicke et al. (2009) and Inoue et al. (2010) including holocephalan taxa relied on 14 and 13 nodes for fossil calibration, respectively. However, many were external to Holocephali, and on a wider taxon sampling across several orders and families of the Chondrichthyes or even on higher vertebrates. For instance, when bird and mammal sequences (plus the corresponding calibration points in these groups) were excluded from the data set by Inoue et al. (2010), their molecular dating yielded generally younger node age estimates. There is further evidence from avian molecular dating studies that the use of external calibration points (e.g. among outgroup lineages) might lead to a considerable underestimation of ingroup node ages (Ho and Larson 2006; Pereira and Baker 2006).

In the present study the split between Elasmobranchii and the modern Holocephali is close to the results of Inoue et al. (2010) (Table 2). Based on these three results it can be assumed that the divergence between the elasmobranchs and the holocephalans was during the Middle Ordovician to the Early Devonian (Fig. 3). The Callorhinchidae diverged from the other two extant families about 187 Ma years ago. Our result intercalates between the results of the former two studies (Heinicke et al. 2009; Inoue et al. 2010) (Table 2). The Callorhinchidae possibly diverged during the Early or Middle Jurassic. In comparison to the branch length of the Callorhinchidae, the extant species are very young, but our age estimation for the three species is older compared to Inoue et al. (2010). Nevertheless, both time estimates were dated to the Miocene (Table 2). The Rhinochimaeridae diverged from the Chimaeridae during the Late Jurassic. Our node time estimates are older than age estimates in the other two studies.

This is the first molecular study that examined the genus *Neoharriotta* including a molecular clock analysis. *Neoharriotta* diverged from *Harriotta* and *Rhinochimaera* during the Middle Cretaceous (Table 2). The result of 47 Ma in Inoue et al. (2010) for the split between *Harriotta* and *Rhinochimaeridae* agrees well with the results of this study. Thus it can be inferred that the two genera diverged during the Eocene. The age for the common ancestor of the Chimaeridae is higher than in Inoue et al. (2010), but a common ancestor can be dated back to the Middle to Late Cretaceous, as for the Rhinochimaeridae. The genus *Chimaera* is estimated to have had a common ancestor about 39 Ma ago (Inoue et al. 2010), but *Chimaera* and *Hydrolagus* are paraphyletic according to our results (see above).

Independent from the time node estimation of molecular studies, the extant holocephalans are seen as living fossils from a clade that dates back to the Palaeozoic (e.g. Patterson 1965; Stahl 1999). The results of the molecular studies offer a good clue to reconstruct their life history and can be compared with new fossil finds. However, at the moment it is hard to reconstruct their life history with certainty so long as the next nearest relatives among other groups of fishes, i.e. Elasmobranchii or Placodermi, remain unresolved.

Conclusions

Concluding the most interesting results, we show here that *Rhinochimaera* is closely related to *Harriotta.* Surprisingly, *Neoharriotta* is the sister species to both. This contrasts with the opinions of internal rhinochimaerid relationships, but may also trigger a new morphological approach in further studies. The second conclusion is that the two supposed genera of Chimaeridae are paraphyletic. Since generic grouping among *Hydrolagus* and *Chimaera* is usually only done on the basis of the absence or presence of the anal fin, a possible multiple loss of the fin might account for the confusion, but this has to be reinvestigated when all species of Chimaeridae are available.

Concerning the systematic distinction of the Genera *Chimaera* and *Hydrolagus* by the character of the absence of the anal fin, it must be stated that the prominent ventral lobe of the tail in *Chimaera monstrosa* is often confluent with the caudal finfold behind and may also be considered a reduced ventral lobe of an originally epicercal caudal fin. The condition in *Chimaera* species probably represents the primitive condition as compared to *Callorhinchus*. Internal structure of this region in different Holocephali still has to be studied in a comparative manner. In any case, it is a reductive character of the species currently placed in *Hydrolagus*, but it is suspected that this is not a very complex and phylogenetically significant clear-cut character to us. Perhaps it would pay to have a closer comparative look at the anatomical details of the species. A further analysis is planned with nuclear genes and promises some progress towards the ingroup relationships of the Holocephali.

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