Clownfishes are a genetic model of exceptional longevity and reveal molecular convergence in the evolution of lifespan

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Abstract

Standard evolutionary theories of aging postulate that reduced extrinsic mortality leads to evolution of longevity. Clownfishes of the genus Amphiprion live in a symbiotic relationship with sea anemones that provide protection from predation. We performed a survey and identified at least two species with lifespan of over 20 years. Given their small size and ease of captive reproduction, clownfishes lend themselves as experimental models of exceptional longevity.

To identify genetic correlates of exceptional longevity, we sequenced the transcriptomes of *Amphiprion percula* and *A. clarkii* and performed a scan for positively-selected genes (PSGs). These were compared with PSGs detected in long-lived mole rats and short-lived killifishes revealing convergent evolution in processes such as mitochondrial biogenesis. Among individual genes, the Mitochondrial Transcription Termination Factor 1 (*MTERF1*), was positively-selected in all three clades, whereas the Glutathione S-

Transferase Kappa 1 (*GSTK1*) was under positive selection in two independent clades.. For the latter, homology modelling strongly suggested that positive selection targeted enzymatically important residues.

These results indicate that specific pathways were recruited in independent lineages evolving an exceptionally extended or shortened lifespan and point to mito-nuclear balance as a key factor.

Keywords

Amphiprion, positive selection, evolution of lifespan, life-history trait, mitonuclear balance

Introduction

The lifespan of vertebrate species spans two orders of magnitude from few months for annual killifish (1) to several centuries for the greenland shark (2). Understanding the genetic architecture underlying these differences is a major challenge but may deliver new insights into the mechanisms controlling evolution of lifespan and human longevity.

Next-generation sequencing technology has revolutionized evolutionary genomics as it allows to obtain genome-scale sequence information for large number of species. A particularly useful approach to identify the genetic architecture of evolutionary novelties is the analysis of positive selection. This approach requires the comparison of the sequence of protein-coding genes in related clades where one of the clades evolved the trait of interest, in this specific case exceptional lifespan. To date, several different mammalian taxa/clades where analysed with this approach with the purpose of identifying sequence changes associated to evolution of longevity: the elephant, the bowhead whale, bats and mole-rats (3-8). These analyses delivered interesting candidate genes and pathways that underwent accelerated molecular evolution in coincidence with evolution of exceptional lifespan. A major drawback

42 of this approach is that these long-living mammals are difficult or impossible to be kept in captivity and
43 manipulated experimentally. This creates the need for a long-lived vertebrate that is small in size, easily
44 adaptable to captive life, can be bred in large numbers and therefore represents a convenient
45 experimental model organism.

46 Standard evolutionary theories of aging predict that low extrinsic mortality conditions lead to the
47 evolution of slow senescence and increased lifespan. Some examples that confirm these theories are the
48 exceptional longevity of vertebrate species under low predation risk since they are chemically protected
49 (9, 10), adapted to an arboreal life (11) or found in protected environments such as caves, respectively.
50 On the other hand, annual fishes of the genus *Nothobranchius* provide an example of how increased
51 extrinsic mortality conditions lead to the evolution of accelerated senescence and short lifespan (12-14).
52 Analysis of positive selection in annual killifishes revealed a potential link between the evolution of
53 genes governing mitochondrial biogenesis and the evolution of lifespan (15).

54 All clownfish species (genus *Amphiprion*) evolved a specific adaptation that allows them to live in
55 symbiosis with sea anemones. Symbiosis evolved in the last common ancestor of clownfish and
56 clownfish represent a monophyletic group in the Pomacentridae family (damselfishes) (16). In the Indo-
57 Pacific Ocean, clownfishes are found in association with one or more sea anemone species and a large
58 variation in host usage exists (17-19). Fish that feel threatened by predators immediately seek
59 protection by the anemone's tentacles; without that symbiosis, fishes are readily attacked and predated
60 (20-22). Therefore, clownfish are protected from predation through reduction of extrinsic mortality
61 owing to the presence of anemones (23). Hence, the overall mortality rate of clownfish is low as
62 compared to other coral reef fishes or other tropical species of Pomacentridae of the same size (20, 23-
63 26).

64 All clownfish are born as males and develop, through protandrous hermaphroditism, into females: in a
65 colony, only the dominant pair contributes to the reproduction of the colony (27).Other individuals of
66 the colony are non-breeding males. Studies in the wild have shown that natural mortality of adult
67 clownfishes can be very low: during the period 2011–2013, the average biannual mortality rate *per*
68 *capita* varied, depending on the study site, between 0.18 and 0.49 for juveniles, 0.09 and 0.44 for males,
69 and 0.19 and 0.55 for females (28). Predatory pressure differs in different stages of adulthood and is
70 increased for non-breeding males (20).

71 These fishes are small in size (less than 10 cm for the smallest species) and the closely-related species *A.*
72 *percula* and *A. ocellaris* are popular and hardy aquarium fishes, are bred in large numbers for the
73 aquarium trade, and are subject to selective breeding to fix specific pigmentation patterns so that a
74 number of different captive strains are available. For these reasons, clownfishes could become the first
75 experimental model for long-living vertebrates.

76 In order to identify the genetic basis of adaptations linked to clownfishes' exceptional lifespans, we
77 performed a positive selection analysis. This analysis requires the identification of the closest related
78 taxon that does not possess the trait of interest in order to exclude events of positive selection that
79 predate the evolution of this trait (29).

80 Other species of damselfish evolved an inter-specific mutualistic relationship with branching corals (30,
81 31). In this case, corals are used by fishes as shelter that can provide protection from predators and a
82 safe area to egg laying (32, 33). Among the family Pomacentridae, *Chromis viridis* shows an interesting
83 relationship with a wide range of scleractinians (34, 35). Despite the presence of favourable
84 microhabitat, *C. viridis* are predated by a wide range of generalist predator species. Hixon and Carr (36)
85 suggested there is a clear relationship among transient and benthic predators and damselfish mortality:
86 damselfish that search for protection in the shelter from transient predators are susceptible to attack by

87 resident benthic predators and vice versa. In the presence of both groups of predators, mortality
88 increases dramatically due to the lack of available refuge that expose *Chromis* to intense predation (36).
89 Therefore, *Chromis viridis* represent a well-suited outgroup for our analysis because it shares with
90 clownfishes several general traits linked to benthic life and symbiosis with corals but it is subject to
91 much higher predation rates (Fig. 1).

92 Captive lifespan of clownfishes

93 In order to obtain a reliable lower estimate for the captive lifespan of clownfish species, in 2016 we
94 distributed a questionnaire to researchers working with clownfishes and to public aquaria across Europe
95 (Table 1/S1), and surveyed existing literature. For six different species, at least one individual was
96 reported to have lived more than 10 years and for two different species, *A. melanopus* and *A. ocellaris*,
97 we obtained record of animals alive and actively spawning at an age of over 20 years. More systematic
98 data could be obtained for the species *A. ocellaris* (the most common species in the aquarium trade).
99 The oldest cohort for which a record was available comprised 27 fish born in 2008 of which 25 were still
100 alive in 2016.

101 We conclude that there is solid evidence that at least the species *A. ocellaris* and *A. clarkii* can live in
102 captivity for more than two decades making them the first teleost model of exceptional longevity.

103 **Table 1.** Results of the clownfish survey. The longest-lived individual for each species is indicated

Species	oldest animal	status at census	size of group
A. akydinos	13	dead	1
*A. clarkii (wild)**	12	alive	n.a.
A. clarkii (privately owned)	16	alive	2/0 dead
A. clarkii	9	alive	2/0 dead
*A. frenatus***	18	dead	n.a.
A. melanopus	21	alive	2/0 dead
A. ocellaris (privately owned)	22	alive	2/0 dead
A. ocellaris	17	alive	2/0 dead

*A. perideraion***	18	alive	n.a.

104 * Moyer, 1986 (37)
105 ** Fautin and Allen, 1992 (17)

106 Analysis of positive selection

107 In order to perform genome-wide scans for positive selection, we obtained the transcriptomes of the
108 species *A. clarkii* and *A. percula* based on own sequencing using methods previously described for the
109 killifishes (15). Furthermore, we assembled clownfish transcriptomes from public read data of *A.*
110 *bicinctus*, *A. ocellaris* and *A. sebae*. As the closest-related non-symbiotic species, we additionally
111 sequenced the transcriptome of *Chromis viridis*, a very abundant species in coral reefs. More distant
112 outgroups were a selection of species from the series Ovalentaria, whose genomes are available in
113 GenBank (see also (15)). We analysed positive selection on the branch leading to the last common
114 ancestor (LCA) of all clownfish species (Fig. 1).

115 A total of 157 positively selected genes (PSGs) of 14214 analyzed genes were identified in the LCA of the
116 clownfishes (Table S2). We tested for overrepresentation of gene ontology (GO, FDR <0.1) and observed
117 19 biological processes enriched for PSGs (Table 2, Table S3). A majority of these processes is of
118 particular interest for aging research: altogether nine enriched processes are linked to the metabolism
119 of xenobiotics, detoxification or glutathione metabolism, respectively. Interestingly, these processes
120 were shown to be strongly up-regulated in experimental conditions favoring longevity such as dietary
121 restriction and inhibition of the somatotropic axis making the animals more resistant to toxins (38-41).
122 Furthermore, experimental manipulation of mitochondrial translation, another enriched process, is
123 known to increase lifespan in *C. elegans* (42).

124 **Table 2.** Biological gene ontology processes enriched for positively selected genes (FDR<0.1).

GOBPID*	Term	FDR**

GO:1901685	glutathione derivative metabolic process	0.020646
GO:1901687	glutathione derivative biosynthetic process	0.020646
GO:0006805	xenobiotic metabolic process	0.08376
GO:0032543	mitochondrial translation	0.08376
GO:0071466	cellular response to xenobiotic stimulus	0.08376
GO:0009410	response to xenobiotic stimulus	0.08376
GO:0042178	xenobiotic catabolic process	0.08376
GO:0007157	heterophilic cell-cell adhesion via plasma membrane cell adhesion molecules	0.08376
GO:0050900	leukocyte migration	0.08376
GO:0045321	leukocyte activation	0.08376
GO:0007155	cell adhesion	0.08376
GO:0022610	biological adhesion	0.08376
GO:0048870	cell motility	0.08376
GO:0051674	localization of cell	0.08376
GO:1990748	cellular detoxification	0.08376
GO:0055081	anion homeostasis	0.08376
GO:0007229	integrin-mediated signaling pathway	0.085657
GO:0016477	cell migration	0.085657
GO:0098754	detoxification	0.085657

* GOBPID – gene ontology biological process ID
* FDR – false discovery rate (adjusted p-value for multiple testing)

Furthermore, mitochondrial translation was one of the mitochondrial biogenesis processes that were found to be enriched for PSGs in extremely short-lived killifishes (15). Recent observations of similar genes and pathways found to be affected by positive selection, both, in very long- and short-lived species led to hypotheses of antiparallel evolution (43, 44). This means that functionally opposite selection pressures with regard to the tradeoff between fast growth and a long lifespan can result in adaptations of the same genes and pathways – in opposite functional directions. We further tested this hypothesis by using Fisher's method to combine enrichment p-values across the results of the recent positive selection analyses in short-lived killifishes and the analysis in clownfishes. In this meta-analysis, 34 genes exhibited a signature of positive selection (FDR<0.1) across species (Tables S4-S6).

Table 3. Positively selected genes associated with mitochondrial biogenesis identified in a meta-analysis across three evolutionary clades with exceptional short or long lifespans.

Gene symbol	p-value				FDR****
	Clownfish LCA*	*Nothobranchius***	Mole-rat**	Combined***	
MTERF1	7.34E-03	1.25E-03	2.57E-02	3.12E-05	4.01E-04
RARS2	1.82E-03	1.18E-02	NA*****	2.52E-04	2.43E-03
MRPL30	2.55E-02	1.00E+00	9.59E-03	2.28E-03	7.98E-03
FASTKD2	1.77E-04	1.56E-01	1.00E+00	3.16E-04	2.43E-03
FASTKD5	NA*****	1.39E-03	8.25E-01	8.89E-03	2.44E-02
TFB2M	NA*****	4.34E-04	6.91E-01	2.73E-03	9.15E-03
NDUFA9	NA*****	8.31E-02	6.90E-03	4.85E-03	1.56E-02

138 * LCA – last common ancestors
139 ** These p-values resulted from meta-analysis using Fisher's method of 3 ancestral Nothobranchius and
140 11 examined mole-rat branches on which lifespan changed considerably
141 *** This p-value results from a meta-analysis of the three p-values in the left columns using Fisher's
142 method
143 **** FDR – false discovery rate (adjusted p-value for multiple testing)
144 ***** NA – no p-value calculated since the gene could not be tested in the respective context

145 An overrepresentation analysis of GO terms among the genes yielded signatures of positive selection in
146 the meta-analysis across different species with exceptional lifespans. As in our previous examination of
147 short-lived killifishes (15), we found an enrichment for mitochondrial biogenesis functions ($p=1.05*10^{-5}$,
148 Table S7). Among the genes involved in mitochondrial biogenesis were *TFB2M* and *MTERF*, that are
149 necessary for mitochondrial transcription, *FASTKD5* and *FASTKD2* whose gene products are required for
150 the biogenesis of mitochondrial ribosomes, (45), as well as *RARS2* coding for a mitochondrial tRNA-
151 synthetase.
152 Among the other 15 PSGs genes showing evidence for positive selection, both, in the clownfish LCA and
153 in meta-analysis were, e.g., *LAMP2* and *CD63* (also called *LAMP3*) which code for major protein
154 components of the lysosomal membrane (46, 47). In addition, *CD63* gene expression was shown to
155 predict the malignancy grade of many different tumor types (48-52) and the artificial prevention of the
156 decrease of *LAMP2* gene expression during aging in mice results in considerably reduced cell damage, as
157 well as in liver functions in old mice that are indistinguishable from those in young mice (53). Finally,
158 another interesting example that was identified as significant, both, in the clownfish LCA and in the

159 meta-analysis, is *GSTK1* encoding a glutathione-S-transferase that localizes to the peroxisome. GSTK1
160 was shown to be associated with diabetes type 2 which is another major aging related disease (54, 55).

161 The positive selection analysis provides not only candidate genes but also candidate amino acids for
162 follow-up studies. To exemplify this, we performed protein homology modeling for GSTK1 starting from
163 the publicly available structures of the human dimeric apoenzyme (PDB 3RPP, (56)) and the rat dimeric
164 enzyme with the bound GSH substrate (PDB 1R4W; (57)). The latter was used to assess on a structural
165 basis the relationship of the six positively selected sites in the clownfish with those that are known to be
166 involved in the enzyme's function (57). Interestingly, also the LCA of Nothobranchius shows positive
167 selection in GSTK1 contains, in addition, one site with high probability of positive selection in the LCA of
168 Nothobranchius (Glu167, blue in Fig.2). The selected site in Nothobranchius, however, is structurally
169 remote to the functionally relevant sites. In contrast, we found that in clownfish two of three sites that
170 were predicted with high probability to be positively selected (≥ 95%, Phe60, Met63, red in Fig.2) and
171 one of three sites with lower probability (41%, His64, orange in Fig.2) belong to the same α-helical
172 stretch of amino acids that lines the substrate access channel, contribute to the dimer interface (Asn61,
173 Tyr65, Asp69, green in Fig. 2) as well as to the substrate binding sites (Lys62, turquoise in Fig. 2),
174 respectively (57). The third site with a high probability to be positively selected is Glu88 (brown in Fig 2).
175 Glu88 is one of four amino acids at the entrance of the substrate access channel and situated in close
176 proximity to Pro55, Pro56 and Pro87 (black in Fig. 2). The latter three are also part of the substrate
177 access channel (57). We found another site positively selected with a lower probability in close proximity
178 to the dimer interface (Lys177, orange in Fig. 2). This positive selection at particular positions related to
179 enzymatic function invites the speculation that it might have a bearing on the enzymatic activity of the
180 clownfish GSTK1, but this hypothesis would have to be tested experimentally.

181 ## Conclusions

182 We have provided evidence for exceptional longevity of clownfishes in captivity. The species *A. ocellaris*
183 is bred in captivity and commercially available in large numbers and we suggest this species as
184 laboratory model for extended lifespan.

185 Analysis of positive selection has shown evolutionary convergence both with the exceptionally short-
186 lived genus *Nothobranchius* and with exceptionally-long lived mole rats.

187 In particular, clownfishes and mole rats both show positive selection in two key proteins of the
188 lysosome: LAMP2 and CD63. These results are consistent with the conserved up-regulation across
189 tissues and species of genes coding for lysosomal proteins and widespread accumulation of lysosomal
190 aggregates observed during aging (58, 59) and suggests that lysosomal function is of key importance for
191 evolution of exceptional longevity. Another interesting example of convergent evolution is GSTK1, which
192 is positively selected in both the exceptionally-long and exceptionally-short lived fish clades. GSTK1 is
193 involved in glutathione metabolism. Since detailed structures of this protein are available (56, 57),
194 homology modelling was possible and it strongly suggests that positive selection targeted positions that
195 are involved in the enzymatic function of the encoded protein.

196 Finally, prominent signs of convergence were observed for genes and pathways related to biogenesis of
197 mitochondrially-encoded proteins with the remarkable observation that MTERF is under positive
198 selection in all three taxa. These findings point to the key importance of mito-nuclear balance in the
199 regulation of animal longevity.

200 # Methods

201 ## Clownfish lifespan estimation

202 The determination of clownfish lifespan was performed through the distribution of an internet-based
203 questionnaire to zoos and aquariums worldwide, requesting information on clownfish demographic
204 details: (1) the various clownfish species maintained in captivity, (2) the number of individuals for each
205 species, (3) if each individual is captive bred or not, (4) the year of acquisition and, if not still alive,
206 death, and (5) the sex of each individual, if determined. The questionnaire was circulated in 2016 to
207 international associations and organizations of zoos and public aquariums such as the European
208 Association of Zoos and Aquaria (EAZA), the Association of Zoos and Aquariums (AZA), the European
209 Union of Aquarium Curators (EUAC) and the World Association of Zoos and Aquariums (WAZA).
210 Responses to our questionnaire were received from 5 zoos and aquariums as well as two private entities
211 (see Acknowledgments).

212 ## Experimental fish and sampling

213 Sub adult *Amphiprion percula* (total length, 45.2±1.2 mm; Wt, 1.6±0.1 g, n=12), *Amphiprion clarkii* (total
214 length, 46.4±5.1 mm; Wt, 2.3±0.9 g, n=12) and *Chromis viridis* (total length, 43.0±1.6 mm; Wt, 1.3±0.1 g,
215 n=12), were used. Animals were acquired from local dealers and subjected to acclimation during one
216 month in the facilities of the Marine Acquarium at the University of Murcia (Spain). Fish were kept in
217 groups under exactly the same conditions (temperature, 27±1 ºC; salinity, 24±1, pH, 8±0.2; dissolved
218 oxygen, 6.5±0.2 mg/L) and fed *ad libitum* four times a day a standard low-fat diet to match their
219 requirements (composed by Mysis shrimp, enriched *Artemia nauplii* and red plankton).

220 Fish were euthanized by exposure to the anesthetic benzocaine hydrochloride (400 mg l-1) for 10 min
221 following the cessation of opercular movement. Brains, livers and samples of skeletal muscle were

222 collected for analyses. For each species, three whole brains were frozen in l-N and stored at -80 ºC prior
223 to molecular determinations.

224 The animal procedures were approved by responsible authorities (A13160603, from the Consejeria de
225 Agua, Agricultura, Ganaderia y Pesca, Comunidad Autonoma de la Region de Murcia, Spain).

226 Coding sequence data

227 Our analysis comprised five clownfish species (*A. ocellaris*, *A. clarkii*, *A. bicinctus*, *A. percula*, *A. sebae*), *C.*
228 *viridis* representing the non-symbiotic sister-taxon of the *Amphiprion* genus and nine more distantly
229 related outgroup species (*Stegastes partitus*, *Pundamilia nyererei*, *Maylandia zebra*, *Oryzias latipes*,
230 *Xiphophorus maculatus*, *Poecilia formosa*, *Fundulus heteroclitus*, *Nothobranchius furzeri*, *Aphyosemion*
231 *striatum*). mRNA sequences of the ougroups were obtained obtained from RefSeq along with their
232 coding sequence annotation (Table S8). For *A. ocellaris*, *A. bicinctus*, *A. sebae* we downloaded read data
233 from the short read archive (Bio projects PRJNA374650, PRJNA261388 and PRJNA285007, respectively).
234 For *A. clarkii*, *A. percula* and *C. viridis* we performed novel RNA-seq as described in Table S9. The reads
235 of the clownfishes and *C. viridis* were preprocessed using SeqPrep with minimum adapter length of five
236 as well as a demanded minimum read length of 50. *De novo* transcriptome assemblies for these species
237 were performed using FRAMA with *Stegastes partitus* as reference species (60). For the clownfishes and
238 *C. viridis* the longest isoform was chosen to represent the gene. For the outgroups, in cases in which
239 multiple isoforms per gene were annotated based on the reference, all of them were used in
240 subsequent analyses. The assembly completeness of all examined species were estimated using BUSCO
241 (61), was 90-100% (Table S8).

242 Identification of positively selected genes

243 To scan on a genome-wide scale for genes under positive selection, we fed the coding sequences of the
244 described species set into the PosiGene pipeline (62). *Stegastes partitus* was used as PosiGene's anchor

245 species. Orthology was determined by PosiGene via best bidirectional BLAST searches (63, 64) against
246 *Stegastes partitus*. The branch of the last common ancestor of the clownfishes was tested for genes
247 under positive selection (Table S2). FDR <0.05 was used as threshold for significance.

248 Gene ontologies

249 We determined enrichments for GO categories using Fisher's exact test based on the R package GOstats
250 (Table S3). The resulting p-values were corrected using the Benjamini-Hochberg method (65). We used
251 throughout the manuscript 0.1 as significance threshold. Enrichment for mitochondrial biogenesis genes
252 was tested using Fisher's exact test and the union set of the genes in the following five mitochondrial
253 related GO terms: GO:0000959, 0032543, 0045333, 0033108, 0070584 (Table S5). The same GO terms
254 were used in our previous study (15) to test for enrichment

255 Meta analysis

256 To identify genes that show signs of positive selection across multiple evolutionary branches on which
257 lifespan was altered considerably, we combined p-values from this study with those of two previous
258 studies using Fisher's method (66) (Table S4-S6). In all three studies, PosiGene was used to determine p-
259 values. The first study searched for genes under positive selection on 11 rodent branches on which the
260 lifespan was presumably extended – most of them in the clade of the African mole-rat family that covers
261 the longest-lived known rodents (67). The second study examined three branches of the *Nothobranchius*
262 genus on which lifespan was presumably reduced (15) – the genus covers the shortest-lived vertebrate
263 species that can be held in captivity (68).

264 Protein homology modeling

265 Homology modelling of the clownfisch GSTK1 was carried out with SWISS–MODEL
266 (http://swissmodel.expasy.org; (69, 70) using the crystal structures of the dimeric apoform of the human

267 mitochondrial GSTK1 (PDB 3rpp; (56)) and the substrate bound dimer of the rat enzyme PDB 1r4w; (57)).
268 No further optimization was applied to the resulting models. Visualisation, superimposition of the
269 respective crystal structures and the models as well as rendering was carried out using CHIMERA (71).

270 Competing interests

271 We have no competing interests.

272 Author contributions

273 AS performed the positive selection analysis and wrote the first draft of the paper, PAP performed the
274 clownfish aclimatation and sampling , MB performed the transcriptome assemblies, MM performed the
275 clownfish survey and helped in writing the paper,′ALS performed the clownfish aclimatation and
276 sampling, JdCR performed the clownfish aclimatation and sampling, MG performed the protein
277 homology modelling, AC concieved and supervised the study, wrote the first draft of the paper. All
278 authors have read and improved the first draft of the paper.

279

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Figures

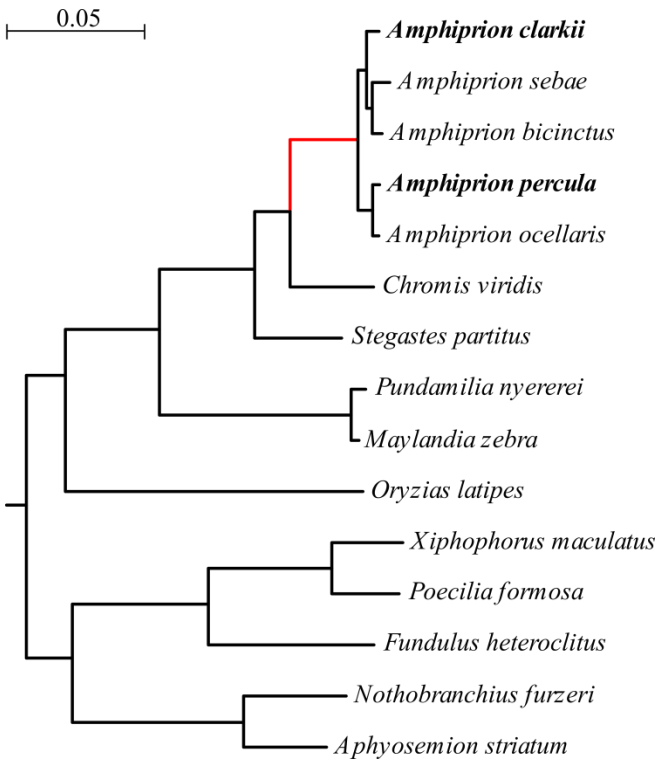


Fig. 1. Nucleotide-based phylogeny of the analyzed fish species. We searched for positively selected genes on the last common ancestor of the clownfishes (*Amphiprion*, red). The two species *A. clarkii* and *A. percula* depicted in bold are those that were sequenced in this study. The phylogenetic tree was derived as part of the positive selection analysis with the PosiGene pipeline (62). Briefly, during this process 8215 genes were concatenated and the resulting concatenated alignment split in 404 fragments each of which had a length of 15 knt. From each fragment, a phylogeny was calculated via maximum

likelihood and, from all resulting 404 trees, a consensus tree was determined using the Phylip package (72). The scale bar represents 0.05 substitutions per site.

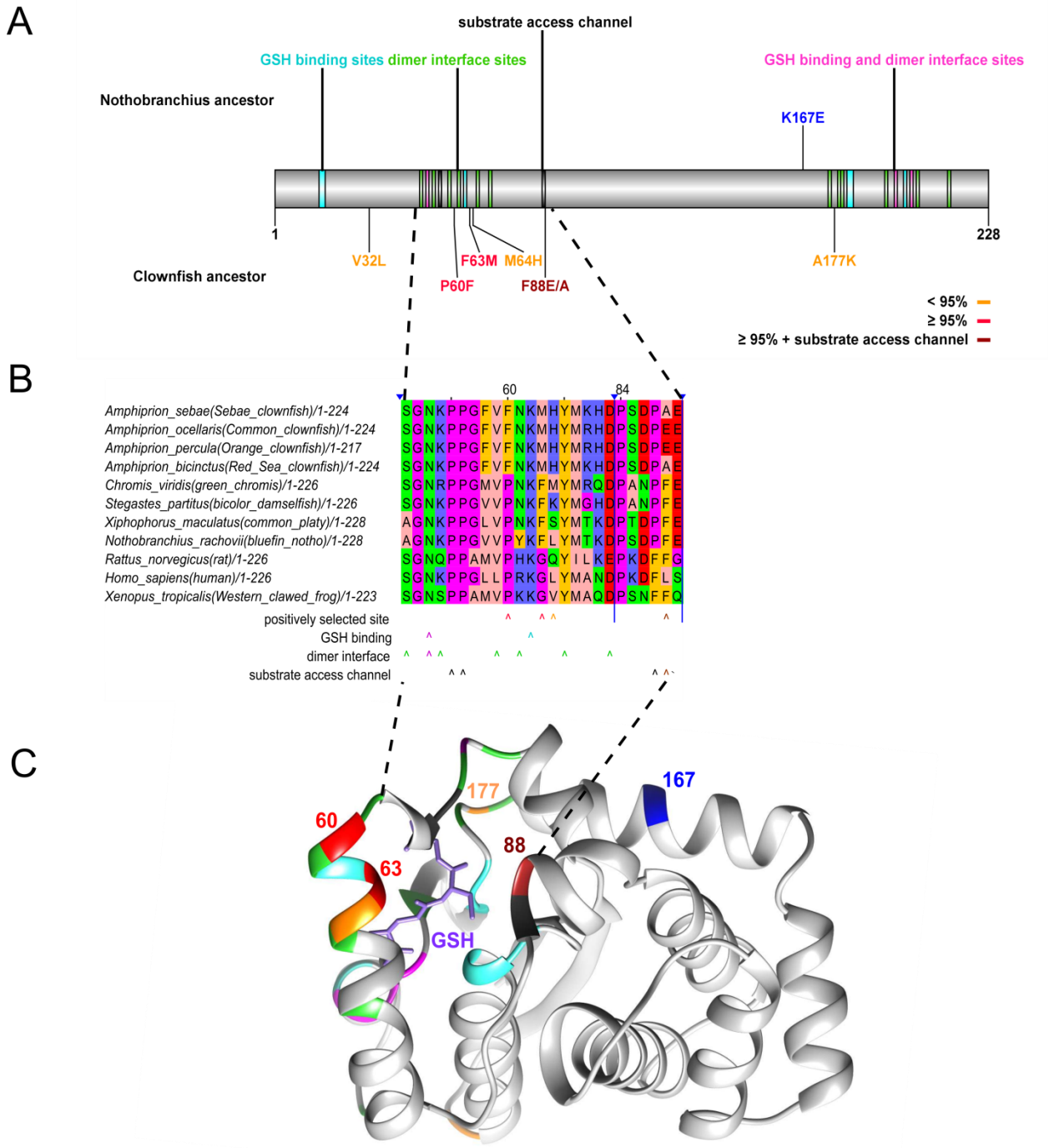
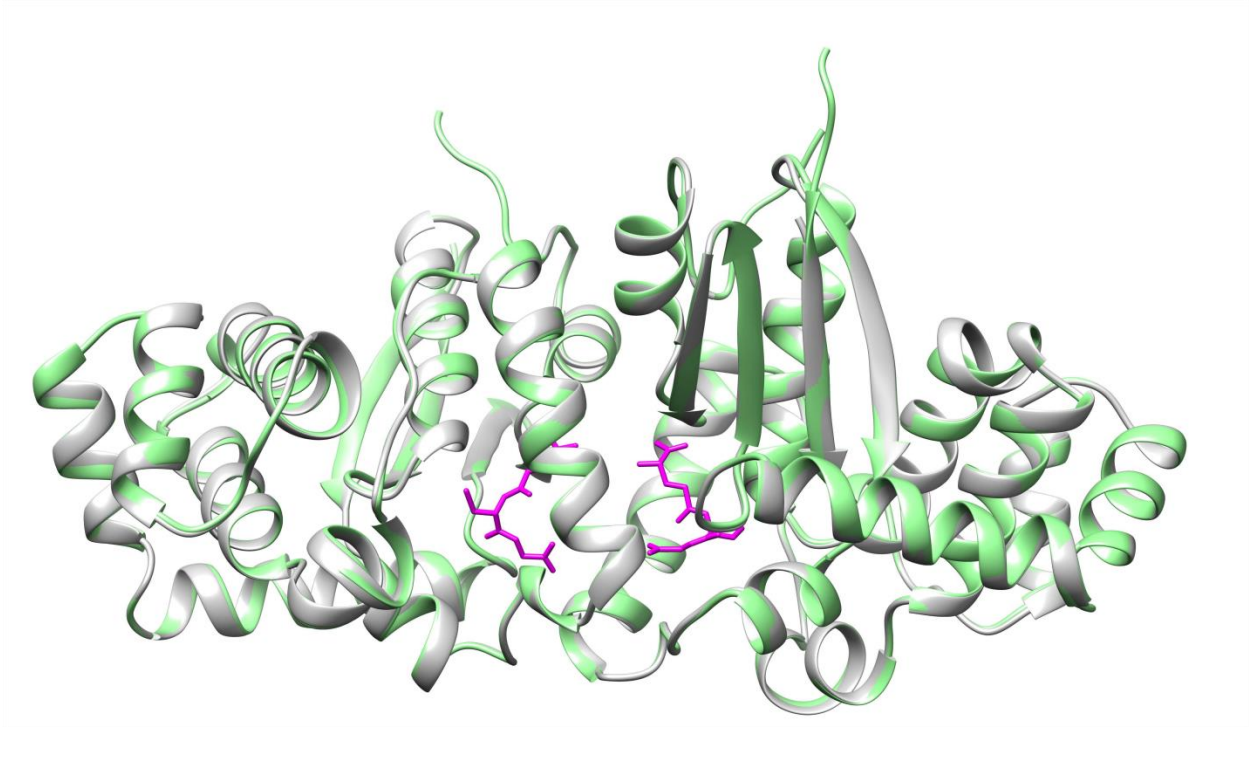


Fig. 2. (**A**) Linear depiction of GSTK1 with color coded known functional domains/sites (dimer interface – green, GSH binding – turquoise, sites that serve as both dimer interface and GSH binding – violet, substrate access channel – black) and positively selected sites (in the last common ancestor of the clownfishes with a predicted probability ≥ 95% – red , in the last common ancestor of the clownfishes with a predicted probability < 95% – orange, in the last common ancestor of *Nothobranchius pienaari* and *Nothobranchius rachovii* – blue. (**B**) Alignment of GSTK1 orthologs across a wide phylogenetic range of species . Depicted are two protein regions (51-69, 84-89) that contain positively selected sites and functionally relevant sites in close proximity. The color code for positively selected and functionally relevant sites is the same as in panel A. (**C**) Clownfish GSTK1 model showing one subunit of the modelled dimer (for an overview see SI Fig S1. Selected positions are color coded according function depicted in the overview scheme at the top. The numbered and colored residue positions (60, 68, 88, 170 and 177) are discussed in detail in the text. Also shown is the GSH substrate (glutathione, light purple) as positioned in the template structure (PDB 1R4W) of the rat GSTK1.



316 **Supplement Fig. S1.** Homology modelling of Clownfish GSTK1. Ribbon representation of the model
317 dimer for the clownfish enzyme as derived from SWISS-MODEL in grey, superimposed onto the dimeric
318 structure of the substrate bound rat GSTK1 (PDB 1r4w; (57)) used as template in light green. The
319 pairwise r.m.s.d. for the Cα positions between the model and 1r4w amounts to 0.52 Å as determined
320 with the CHIMERA Matchmaker tool. The GSH substrate in the rat enzyme structure is rendered in light
321 purple.

322 # Supplement

323 ## Supplement tables

324 **Table S1.** Clownfish lifespan questionnaire results.

325 **Table S2.** PosiGene results for positively selcted genes on the phylogenetic branch representing the last
326 common ancestor of the the clownfishes (genus *Amphiprion*).

327 **Table S3.** Enrichment test resullts of biological gene ontology processes enriched for positively selected
328 genes.

329 **Table S4.** Meta analysis using Fisher's method of positive selection across three analyses of phylogenetic
330 branches on which lifespand changed considerably.

331 **Table S5.** Meta analysis using Fisher's method of positive selection across three phylogenetic branches
332 of the Nothobranchius genus on whichlifespan was reduced considerably.

333 **Table S6.** Meta analysis using Fisher's method of positive selection across eleven phylogenetic rodent
334 branches on which lifespan was reduced considerably.

335 **Table S7.** Genes that were regarded as mitochondrial biogenesis related from five gene ontology terms.

336 **Table S8.** Assembly and sequence statistics.

337 **Table S9.** Samples that were sequenced to create genome/transcriptome assemblies.

338 Supplement data

339 ftp://genome.leibniz-fli.de/pub/user/arne.sahm/clownfish/supplement_data.tar.gz

340 The package contains assembled sequence data, visualizations of alignments and positively selected

341 sites for all genes that were analyzed in this article.

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