BRIEF COMMUNICATION

Functional conservation and divergence of color-pattern-related agouti family genes in teleost fishes

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Abstract
While color patterns are highly diverse across the animal kingdom, certain patterns such as countershading and stripe patterns have evolved repeatedly. Across vertebrates, agouti-signaling genes have been associated with the evolution of both patterns. Here we study the functional conservation and divergence by investigating the expression patterns of the two color-pattern-related agouti-signaling genes, agouti-signaling protein 1 (asip1) and agouti-signaling protein 2b (asip2b, also known as agrp2) in Teleostei. We show that the dorsoventral expression profile of asip1 and the role of the "stripe repressor" asip2b are shared across multiple teleost lineages and uncover a previously unknown association between stripe–interstripe patterning and both asip1 and asip2b expression. In some species, including the zebrafish (Danio rerio), these two genes show complementary and overlapping expression patterns in line with functional redundancy. Our results thus suggest how conserved and novel functions of agouti-signaling genes might have shaped the evolution of color patterns across teleost fishes.

KEYWORDS
agouti gene family, asip1, asip2b, coloration, pigmentation, Teleostei

1 | INTRODUCTION

Pigment patterns such as countershading and stripes have evolved repeatedly across vertebrates and serve as antipredatory strategies (Barlow, 1972; Cott, 1940; Kapp et al., 2018; Thayer, 1909). In the last decade the genetic basis of some color patterns and mutations driving their phenotypic variation have been identified in vertebrates (Manceau et al., 2010; Mills & Patterson, 2009). One of the molecular pathways that has been repeatedly associated with coloration phenotypes is the melanocortin system (Candille et al., 2017; Eizirik et al., 2003; Gross et al., 2009). When bound by the alpha-melanocyte-stimulating hormone (α-MSH), the melanocortin receptor 1 (Mc1r) promotes pigment cell proliferation or pigment biosynthesis and transport, yet this pathway can be blocked by agouti-signaling proteins that act as antagonists. Coding mutations in the melanocortin receptor often cause changes in overall pigmentation (see e.g., Rosenblum et al., 2010), regulatory variations in the agouti-signaling gene, on the other hand, are often linked to the differences in pigment patterns (see e.g., Manceau et al., 2011). For example, the gene agouti-signaling protein (Asip/asip1, gene name in tetrapods/fish) has been shown to underly changes in dorso-ventral countershading (Cal et al., 2017; Ceinos et al., 2015; Linnen et al., 2013; Manceau...
et al., 2011). Recently, Asip also has been linked to stripe patterns in tetrapods (Haupaix et al., 2018; Mallarino et al., 2016), where high expression in the light areas between melanic stripes might locally inhibit dark pigmentation and thereby shape the pattern. More recently, a paralog of Asip/asip1, the teleost-specific agouti-signaling protein 2b (asip2b, in previous literature mostly referred to as agrp2; here we only use the gene name asip2b throughout as it is justified by the gene tree and paralogy/orthology relationships in that gene family as previously discussed [Braasch & Postlethwait, 2011]; Figure 1), has been shown to inhibit stripe patterns in East African cichlids, however not by spatial expression differences but by acting as a global inhibitor of stripe patterns (Kratochwil et al., 2018; Kratochwil, 2019). Other members from the agouti gene family including agrp1 and asip2a neither show substantial expression in the vertebrate skin nor have been previously implicated in pigment pattern formation (Kurokawa et al., 2006; Sanchez et al., 2010; Song & Cone, 2007).

Here, we investigate the functional conservation and diversity of the color-pattern-related agouti-signaling genes asip1 and asip2b in color patterns formation and presence/absence across the largest vertebrate radiation: the teleost fishes, Teleostei. The aim was to investigate three questions: (1) To what extent is the expression pattern of Asip/asip1 in shaping dorso-ventral countershading and stripe patterns conserved across the phylogeny? (2) Was the previously reported role of asip2b in inhibiting stripe patterns repeatedly coopted during teleost fish evolution? (3) Do the paralogs asip2b and asip1 have redundant or complementary functions and thereby provide some initial insights into more ancestral mechanism of color pattern formation in vertebrates?
2 | MATERIALS AND METHODS

2.1 | Animals

Fishes used in this study were from commercial breeders and kept in the animal facility at the University of Konstanz. All animal samples used in this study were collected in accordance with relevant guidelines and regulations and sampling was approved by the authorities (Regierungspräsidium Freiburg, Anzeige T-16/13).

2.2 | Phylogenetic and synteny analysis of agouti gene family

To construct a revised phylogeny of the agouti gene family we used annotated sequences (Supporting Information Data) from ensemble genomes and the NCBI database and used the NGPhylogeny pipeline (Lemoine et al., 2019) with the standard settings. Sequences were aligned using MAFFT v7.407 (Katoh & Standley, 2013). To select phylogenetically informative regions, we used the BMGE v.1.12 (Block Mapping and Gathering with Entropy) software (Lefort et al., 2017). Synteny was investigated using the ENSEMBL genome browser (Release 100).

2.3 | RNA extraction and quantitative real-time PCR (RT-qPCR)

RNA extraction, complementary DNA (cDNA) synthesis and RT-qPCR were performed as previously described (Kratochwil et al., 2018; Liang et al., 2020). Briefly, dissected skin tissues were stored in RNAlater (Invitrogen). RNA was extracted using TRIzol (Invitrogen) and purified by RNeasy Mini Kit (Qiagen). On-column DNase treatment was performed with RNase-Free DNase Set (Qiagen). First strand cDNA was synthesized using the GoScript Reverse Transcription System (Promega). Quantitative polymerase chain reactions (qPCRs) were performed in 20 µl reaction with first strand cDNA, forward and reverse primers and GoTaq qPCR Master Mix (Promega). Primers for qPCR are listed in Table S1. We used 40 cycles of amplification on a CFX96 Real-Time PCR Detection System (Bio-Rad). The amplification program was: Initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 20 s, 60°C for 60 s. At the end of the cycles, melting curve of the products was verified for the specificity of PCR products. Gene expression was assayed in triplicate for each sample and the relative expression between samples was compared using the 2<sup>−ΔΔCT</sup> method (Nolan et al., 2006). For group comparison, we used analysis of variance (ANOVA) followed by Tukey’s honestly significant difference (HSD) test. All statistical tests were performed in R (R Development Core Team, 2011).

3 | RESULT

3.1 | Phylogenetic and molecular evolutionary analyses of agouti-signaling genes

To investigate the molecular evolution of the agouti gene family, we constructed a revised phylogenetic tree that incorporated several sequences from sharks, rays, nonteleost fishes as well as an agrp-like gene from hagfish (Figure 1a and Figure S1). Similar to previous phylogenies (Braasch & Postlethwait, 2011), agouti gene family members cluster into four groups: Asip/asip1 orthologs, agouti-related peptide (Agrp/agrp1) orthologs and the a2 group which contains both the nonteleost asip2, and the teleost-specific asip2a and asip2b paralogs that arose through the teleost specific genome duplication (TSGD). An evident feature of the agouti family phylogeny is the high frequency of gene losses with several ohnologs gone missing (Figure 1a and Figure S1). Based on the tree and as suggested previously (Braasch & Postlethwait, 2011), proto-agrp and proto-asip originated from an ancestral agouti precursor gene at the first (R1) whole-genome duplication (WGD). In the second WGD (R2), proto-asip gave rise to Asip/asip1 and asip2. Interestingly, the gene asip2 can be neither found in lamprey, hagfish, cartilaginous fishes nor tetrapods and was only retained in the Actinopterygii. The TSGD (Hoegg et al., 2004; Steinke et al., 2004) resulted in the two paralogs asip2a and asip2b in Teleostei. However, also with our revised phylogeny the outstanding question, whether asip2 is the paralog of agrp1 or asip1, remains still debatable (Schioth et al., 2011).

Next, using several recently sequenced nonteleost fish genomes, we performed synteny analyses to provide additional confirmation for the phylogenetic relationships of asip1, asip2, asip2a, and asip2b. The synteny of the asip1 locus was largely conserved across Actinopterygii (Figure 1b). Similar results could be obtained from synteny analyses of nonteleost fish asip2, teleost specific asip2a and asip2b (Figure 1c-e). Interestingly, the direction of asip1 in Lepisosteus oculatus, Scleropages formosus, Danio rerio, Myripristis murdjan is inverted if compared to Betta splendens, Takifugu rubripes and Oreochromis niloticus. Comparison between asip1 and the a2 group genes revealed that no neighboring genes are shared between the paralogs (Figure 1b-d). However, when comparing the neighboring genes of teleost fish asip2a and asip2b with asip2 in nonteleost Actinopterygii (Figure 1c-e), we found that adjacent genes of both asip2a and asip2b shared conserved synteny with nonteleost Actinopterygii in some extent: both asip2 and asip2a are flanked by snx16 (sorting nexin-16) on the 5' side and wwp1 (WW domain containing E3 ubiquitin protein ligase 1) and rmdn1 (regulator of microtubule dynamics 1) on the 3' side, while atp6v0d2 (v-type proton ATPase subunit d 2) can be found in the 3' side of both asip2 and asip2b. Thus, the synteny results confirm previous data that suggested that teleost asip2a and asip2b originated from asip2 during the TSGD (Braasch & Postlethwait, 2011).
FIGURE 2  Expression differences of asip1 and asip2b associate with stripe pattern formation and evolutionary loss across teleost fishes. (a) Phylogenetic relationship of focal striped and nonstriped species of this study. Melanochromis auratus and Pseudotropheus cyaneorhabdos are two striped cichlids from East African cichlid radiations. Nonstriped Nanochromis parilus and striped Pelvicachromis pulcher are two West African cichlids. Trichromis salvini is a Neotropical cichlid fish. Note that all focal striped cichlids have two stripes. Betta pi is an anabantoid fish that also belongs to the modern teleosts (compared to the more basal zebrafish) with three horizontal stripes. Danio tinwini (gold-ring danio) is a species of the genus Danio from Myanmar with melanic spot patterns and Danio rerio (zebrafish) is a model organism and has five horizontal stripes—both belong to the carp subfamily Danioninae. Divergence times were taken from (Hughes et al., 2018; Kumar et al., 2017). (b–g) Horizontal stripe patterns and the spatial expression of asip1 and asip2b for M. auratus (b), Ps. cyaneorhabdos (c), Pe. pulcher (d), T. salvini (e), B. pi (f) and D. rerio (g). Left panels, skin of striped species showing the characteristic stripe patterns; Middle panels, spatial expression ratio of asip1 along dorsoventral axis; Right panels, spatial expression ratio of asip2b along dorsoventral axis. The p values are based on ANOVA and Tukey–Kramer post hoc tests (full data see Tables S2–S7). Error bars indicate means + SD. ***p < .001; **p < .01; *p < .05. (h) Expression differences of asip1 in the species with two stripes. The p values are based on ANOVA and Tukey–Kramer post hoc tests (full data see Table S8). Each dot represents the mean value of one species. Black/gray lines depict the mean of the four species. ***p < .001; **p < .01; *p < .05. (i, j) Whole skin expression comparison of asip2b of nonstriped and striped species: nonstriped N. parilus versus striped Pe. pulcher (i) and nonstriped D. tinwini versus striped D. rerio (j). Differences in (i, j) were tested by two-tailed t test, n = 6 in (i) and 5 in (j) (individual dots). Error bars indicate means + SD. ***p < .001; **p < .01; *p < .05. Abbreviations: DIN, dorsal interstripe; DLS, dorsolateral stripe; DOR, region dorsal to dorsal-most stripe; expr, expression; MLS, midlateral stripe; VEN, region ventral to ventral-most stripe; VIN, ventral interstripe; VLS, ventrolateral stripe [Color figure can be viewed at wileyonlinelibrary.com]
3.2 | The gene asip1 is associated with countershading and stripe patterns

Previous studies have shown that expression of asip1 varies along the dorso-ventral axis among teleost fishes (Ceinos et al., 2015; Cerda-Reverter et al., 2005; Guillot et al., 2012; Kurokawa et al., 2006), while it is unknown if asip1 is generally associated with stripe patterns in teleost as it is in tetrapod (Haupaix et al., 2018; Mallarino et al., 2016). To address the role of asip1 across teleost fishes, we performed qPCRs to examine spatial expression differences (a) along the dorso-ventral axis and (b) between melanic stripes (referred to as stripe) and nonmelanic-stripe (referred to as interstripe) regions of the integument from different lineages of striped teleost fishes (Figure 2a), including two East African cichlids (Melanochromis auratus and Pseudotropheus cyaneorhabdos), a West African cichlid (Pelvicachromis pulcher), a Neotropic cichlid (Trichromis salvini), and an Anabantoid fish (Betta pi) and a more distantly related teleost, the zebrasfiish (D. rerio).

In general, asip1 showed differential expression in all tested species (ANOVA: all p < .01; Tables S2–S7). Compatible with the role of asip1 in countershading, for all species the lowest expression was found in the most dorsal regions (region dorsal to dorsal-most stripe [DOR], or dorsolateral stripe [DLS]) while the highest expression levels of asip1 were found in the most ventral areas (VEN) (Figure 2b–g and Tables S2–S7). However, the asip1 expression did not continuously increase from dorsal to ventral but was found to be generally higher in nonmelanic regions than in the adjacent melanic stripes (in most species and across the dorso-ventral axis). Because of the relatively low expression values in dorsal regions (and therefore higher between-sample variation) expression differences were typically not significant (Figure 2b–g and Table S2–S7). But when performing statistical tests by combining species-specific mean values of the four species with two stripes (M. auratus, Ps. cyaneorhabdos, Pe. pulcher and T. salvini; Figure 2b–e), we found asip1 expression to be significantly higher (3.6–9.4 times) in the dorsal interstripe (DIN) than in the adjacent stripes (DLS and midlateral stripe [ML5]) (Tukey HSD: p = .008–.032; Figure 2h and Table S8). Interestingly, asip1 expression was also slightly elevated in interstripe regions of B. pi, but mainly in the dorsal regions of the integument (Figure 2f and Table S6). In contrast, such elevation of asip1 messenger RNA (mRNA) levels in interstripes was only found in the more ventral regions of D. rerio (Figure 2g and Table S7). Thus, our results confirm a conservation of the dorso-ventral asip1 expression gradient and suggest a remarkably conserved striking stripe-interstripe differential expression in teleosts and that also—interestingly, based on previous findings (Haupaix et al., 2018; Mallarino et al., 2016)—seems to apply to vertebrates more generally.

3.3 | The gene asip2b might function as “stripe repressor” across teleost fishes

Another agouti-signaling gene, asip2b, has been recently shown to facilitate loss of stripe patterns in East African cichlid fishes: high expression of asip2b blocks formation of stripe, while low expression permits stripe presence. Expression of asip2b across skin is hereby suggested to act as a global switch of stripe patterns (Kratchowil et al., 2018; Liang et al., 2020). What remains unclear is, whether this function of asip2b is specific to East African cichlids or is found also generally in other lineages of the teleost radiation. To test this hypothesis, we identified species pairs that are closely related and differ in stripe presence/absence: two West-African cichlids of the subfamily Pseudocrenilabrinae, the striped Pe. pulcher and the nonstriped Nanochromis parilus (the divergence time to East African, haplochromine cichlids is about 25 million years; Genner et al., 2007; Schwarzer et al., 2014), and two relatives of the zebrasfiish in the subfamily Danioninae, the striped D. rerio and the nonstriped Danio tinwinii (McCluskey & Postlethwait, 2015) (Figure 2a). Comparative analysis of asip2b skin expression using qPCR revealed similar patterns as those that had been described for East African cichlids (Kratchowil et al., 2018): within both Pseudocrenilabrinae and Danioninae asip2b mRNA levels were significantly higher in the nonstriped species than in the striped species (pairwise t test, p < .001 in Pe. pulcher vs. N. parilus, p < .05 in D. rerio vs. D. tinwinii; Figure 2i,j and Tables S9 and S10). Thus, stripe presence-absence is repeatedly associated with asip2b expression variation across cichlid lineages and in the distantly related teleost fishes of the subfamily Danioninae, suggesting that asip2b might function as global stripe repressor not only in in East African cichlids, but also in other teleost lineages.

3.4 | Functional redundancy of asip2b and asip1

While it has been strongly suggested for the East African cichlid fish Haplochromis sauvaei that asip2b only controls absence/presence but likely not positioning of stripes (Kratchowil et al., 2018), a role for stripe positioning has not been tested for other striped cichlids and teleost fishes. Therefore, we wanted to test if asip2b might show spatial expression variation in teleost fishes (similarly as observed for asip1). Based on qPCRs for asip2b performed in the same manner as we did for asip1 we found no expression differences across the dorso-ventral axis in M. auratus, Ps. cyaneorhabdos, Pe. pulcher and B. pi (ANOVA: p = .168–.932; Figure 2b–d and f, and Tables S2–S4 and S6), indicating that asip2b does—as previously suggested (Kratchowil et al., 2018; Liang et al., 2020)—likely play no role in shaping the horizontal stripe patterns in these species. In contrast, in the Neotropic cichlid T. salvini, asip2b mRNA levels were higher in nonmelanic regions than in melanic stripes (ANOVA: p < .01; Figure 2e and Table S5), similar as observed for asip1. This might suggest that this expression pattern is ancestral for the cichlids and was later modified in some East African lineages. The expression variation of asip2b in zebrasfiish is even more intriguing. We observed expression differences between stripes and interstripes (ANOVA: p < .001; Figure 2g and Table S7). However, stripe–interstripe differences in asip2b expression are restricted to the dorsal parts. This is complementary to the
stripe–interstripe differences in asip1 expression that we found in ventral areas suggesting complementary functions of asip2b and asip1. Our results therefore suggest a dual role for asip2b for controlling both, stripe presence/absence and stripe positioning in some but not all investigated teleost lineages.

4 | DISCUSSION

Here, we investigate the expression patterns of the color-pattern-related agouti-signaling proteins, asip1 and asip2b in relation to dorsoventral and stripe patterns across several teleost lineages. We suggest that both asip1 and asip2b each evolved multiple functions in color pattern formation with asip1 having a conserved role for both dorsoventral patterning and stripe patterning and asip2b for stripe patterning and stripe presence/absence (by acting as a general inhibitor).

The agouti family gene asip1 is consistently associated with dorsoventral countershading and stripe patterns. This is in line with previous findings that have shown dorsoventral expression differences of asip1 in zebrafish (Ceinos et al., 2015) and flatfishes (Guillot et al., 2012), and asip in the nonteleost spotted gar (L. oculatus) (Cal et al., 2017), as well as of the Asip in tetrapods (Linnen et al., 2009; Manceau et al., 2011). Expression differences between melanic stripes and bright interstripes have previously only been reported for tetrapods (Haupaix et al., 2018; Mallarino et al., 2016). The similar patterns we described within teleost fishes in this study, suggesting that asip1 has been repeatedly recruited as part of the molecular mechanism controlling striped patterns.

A second agouti family gene, asip2b, has been previously shown to underly the repeated evolutionary losses and gains of stripe patterns in East African cichlid fishes. Here we show that the role of asip2b in blocking stripe pattern formation, as reported in East African cichlids (Kratochwil et al., 2018), might have been repeatedly coopted during teleost fish evolution. In two stripe-nonstriped species pairs, one pair of Pseudocrenilabrinae and one pair of Danioni- nae, we find the same pattern of differential expression with the nonstriped species having higher asip2b expression (Figure 2i). More comparisons with a larger taxonomic breadth are necessary to more strongly support this hypothesis, but pairs of closely related species (that also allow to use the same qPCR primers), one with and one completely without stripes are scarce and are often not ideal (as in the case of D. tinwini that still has a spot pattern that might or might not be affected by asip2b as one might argue).

While previous work (Kratochwil et al., 2018; Liang et al., 2020) suggested that asip2b is rather ubiquitously expressed across the whole skin, we identified spatial expression variation in two species that resemble the patterns observed for asip1. It is likely that asip2b have retained some properties of Asip/asip1 (that itself remained functionally highly conserved across vertebrate evolution) including the skin-specific expression, and, in some lineages, expression in interstripes and the ventral integument, for example, asip2b shows higher expression in the nonmelanic regions between the stripes and in the ventral part in Neotropical cichlid T. salvini. What is conserved regarding the expression in nonmelanic regions might be however not the exact dorso-ventral position(s) (as at least stripes are most likely not the ancestral condition), but the gene-regulatory interactions that orchestrate the upregulation of agouti genes as melanocortin receptor antagonists to facilitate pattern formation. Based on the lack of skin-specific expression (and the lack of any reports supporting a role in color pattern formation) of the other two agouti family members Agrp/agrp1 and asip2a we furthermore speculate that the evolution of the skin-specific role evolved after the first genome duplication event (R1; Figure 1) and that skin-specific expression has been lost in asip2a after or through the TSGD event (Figure 1).

In D. rerio, asip2b shows higher expression in DINs, implying a possible subfunctionalization of asip1 and asip2b into ventral and DIN-expression domains, such compensatory effects might also possibly explain, why knockouts of asip1 alone did not lead to any effects on zebrafish stripe patterns (but only on dorsoventral patterning) (Cal et al., 2019), while an asip2b/asip1 double knockout could result in stripe pattern defects—a hypothesis that should be tested in the future. Moreover, asip2b also underwent neofunctionalization by evolving, potentially even repeatedly, into a “stripe-repressor” within cichlids and—as suggested here—also in other lineages.

In summary our comparative study on the evolutionary dynamics of agouti gene family expression and function provides new insights that demonstrate how Asip/asip1 remained remarkably constrained and conserved in its expression pattern, asip2b retained functional properties of Asip/asip1 while the gene at the same time seem to have repeatedly sub- and neofunctionalized to shape and facilitate the evolution of stripe patterns across teleost fishes.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available in the supplementary material of this article.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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