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., 2007; 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
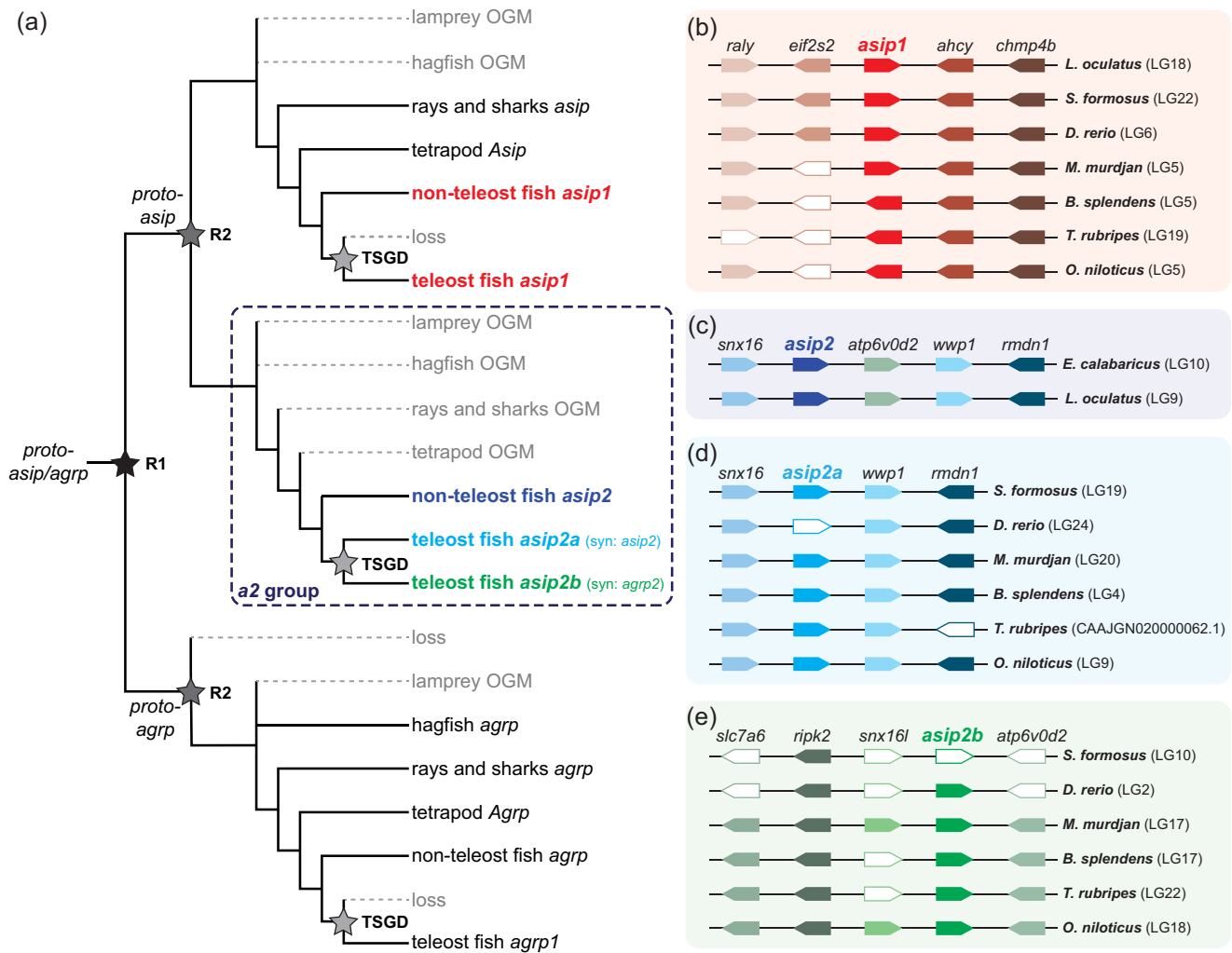
FIGURE 1 Molecular evolutionary analyses of the agouti gene family across vertebrate. (a) Simplified model of agouti gene family evolution based on an updated maximum likelihood phylogeny (Figure S1) including suggested gene loss and OGM events (based on available genomes). Please note that it still remains questionable whether *asip2* is the paralog of *agrp1* or *asip1*. Also, the timing of the genome duplications in early vertebrates and if they are shared between hagfishes, lampreys and other vertebrates is still in debate. (b–e) Chromosomal segments showing the synteny of *asip1* (b), *asip2* (c), *asip2a* (d) and *asip2b* (e) in nonteleost fishes and teleost fishes. Different genes are represented by different colored pentagons and the gene order is determined by their relative positions in the chromosome/scaffold. The direction of the pentagons indicates the gene direction. Hollow pentagons represent gene missing/not found. Abbreviations: R1, R2: vertebrate genome duplication round 1 and 2; OGM, ohnolog gone missing; TSGD, teleost specific genome duplication [Color figure can be viewed at wileyonlinelibrary.com]

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 (Criscuolo & Gribaldo, 2010). To calculate the maximum-likelihood phylogeny, we used PhyML 1.8.1 (Guindon et al., 2010) with Smart Model Selection (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 previous 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 qPCRMaster 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 $2^{-\Delta\Delta CT}$ 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., 2006) 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).
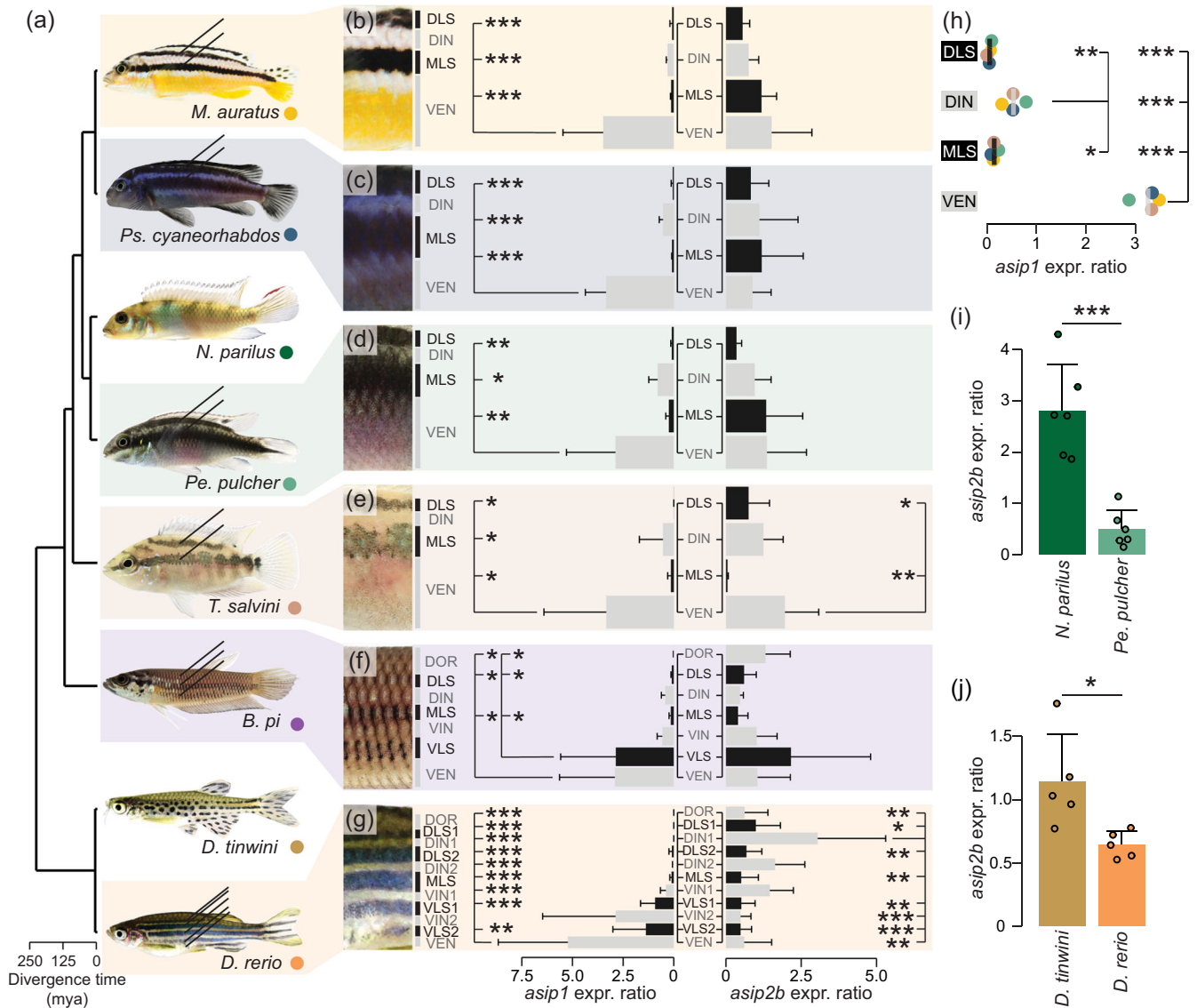
FIGURE 2 Expression differences of *asip1* and *asip2b* associate with stripe pattern formation and evolutionary loss across teleost fishes. (a) Phylogenetic relationship of focal striped and nonstriped species of this study. *Melanochromis auratus* and *Pseudotropheus cyaneorhabdos* are two striped 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 < .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, ventralateral 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*), an Anabantoid fish (*Betta pi*) and a more distantly related teleost, the zebrafish (*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 [MLS]) (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 (Kratochwil 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 zebrafish in the subfamily Danioninae, the striped *D. rerio* and the nonstriped *Danio tinwini* (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 (Kratochwil 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. tinwini*; 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 sauvagei* that *asip2b* only controls absence/presence but likely not positioning of stripes (Kratochwil 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* = .168–0.932; Figure 2b–d and f, and Tables S2–S4 and S6), indicating that *asip2b* does—as previously suggested (Kratochwil 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 zebrafish 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 Danioninae, we find the same pattern of differential expression with the nonstriped species having higher *asip2b* expression (Figure 2i,j). 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.

CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

PEER REVIEW
The peer review history for this article is available at https://publons.com/publon/10.1002/jez.b.23041

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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