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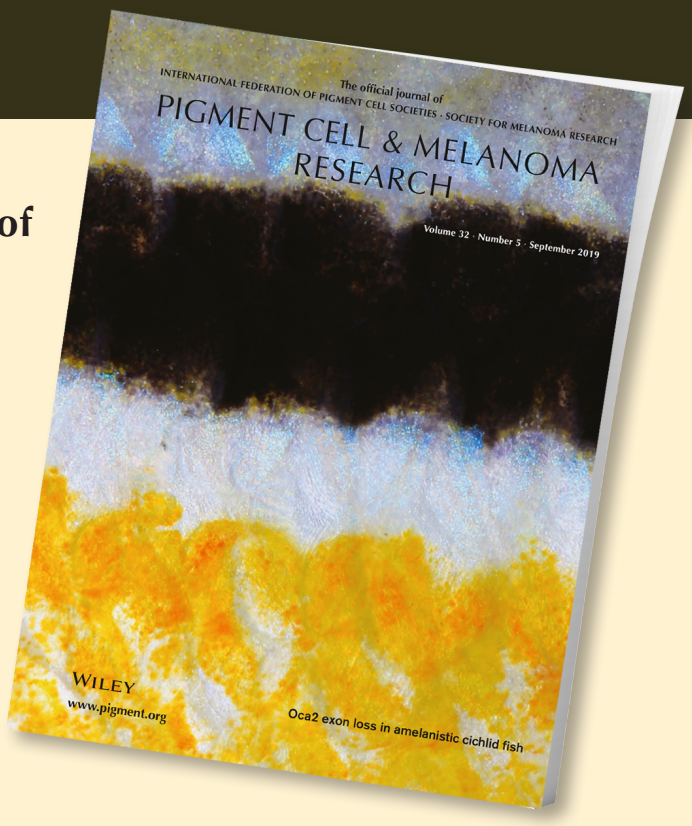

Genome of the Malawi golden cichlid fish (*Melanochromis auratus*) reveals exon loss of *oca2* in an amelanistic morph

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SHORT COMMUNICATION

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Genome of the Malawi golden cichlid fish (*Melanochromis auratus*) reveals exon loss of *oca2* in an amelanistic morph

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Abstract

The tropical freshwater fish family Cichlidae is famous for its record-breaking rates of speciation and diversity in colors and color patterns. Here, we sequenced the genome of the Lake Malawi cichlid *Melanochromis auratus* to study the genetic basis of an amelanistic morph of this species that lacks the typical melanic stripes and markings. Genome sequencing of the amelanistic and wild-type morph revealed the loss of the second exon of the known pigmentation gene *oculocutaneous albinism II* (*oca2*), also known as *p(ink-eyed dilution) gene* or *melanocyte-specific transporter gene*. Additional genotyping confirms the complete association with this recessive Mendelian phenotype. The deletion results in a shorter transcript, lacking an acidic di-leucine domain that is crucial for trafficking of the Oca2 protein to melanosomes. The fact that *oca2* is involved in a wide range of amelanistic morphs across vertebrates demonstrates its highly conserved function.

KEYWORDS
albinism, amelanism, Cichlidae, coloration, teleost fishes

Cichlid fishes are among the most species-rich and most colorful fishes. With their over 2,500 species, they are a textbook model system for studying speciation and adaptation (Henning & Meyer, 2014; Kratochwil & Meyer, 2015; Salzburger, 2018). In recent years, several conspicuous cichlid coloration phenotypes such as stripe patterns, egg spots, and the orange blotch phenotype have been genetically dissected, providing novel insights into color pattern formation and evolution more generally (Ahi & Sefc, 2017; Kratochwil et al., 2018; Roberts, Ser, & Kocher, 2009; Salzburger, Braasch, & Meyer, 2007; Santos et al., 2014). Cichlids are also very popular fish among hobbyists that breed and maintain species and color morphs. Some of the most noticeable pigmentation abnormalities that can be found in cichlids and vertebrates in general are amelanistic and albinistic morphs. Amelanism results from several types of genetic changes that affect melanin, which is synthesized in specialized cells called melanocytes (in mammals and birds) or melanophores (e.g., in reptiles, amphibians, and teleost fish; Manga, 2018; Saenko et al., 2015). Albinistic individuals (although inconsistently defined across different vertebrate lineages) do not only lack the brown or black melanin-based color patterns but also other pigments including, for example, the orange to red pheomelanin that can be also synthesized in mammalian and avian melanocytes or yellow to red pteridines and carotenoids that are contained in xanthophores and erythrophores of teleost fishes (Schartl et al., 2016).

The Malawi golden cichlid or golden mbuna (*Melanochromis auratus*) is a cichlid fish endemic to Lake Malawi. The basic coloration of wild-type (WT) *M. auratus* females and submissive males (Figure 1a) is light yellow to gold, with two melanic horizontal stripes (midlateral stripe; mls and dorsolateral stripe; dls), flanked by white coloration (Figure 1e). The ventral side usually shows a stronger yellow coloration (Figure 1e) with intensity varying between individuals. Variation in yellow coloration is not linked to the amelanistic phenotype. Dominant males of *M. auratus* are brown to black (Figure 1b). In the same location where melanic stripes are located in females, the dark basic coloration is interrupted by two silvery to bluish horizontal stripes (Figure 1g). Both, females and dominant males, have

Kratochwil and Urban contributed equally to this work.

a strongly pigmented iris, showing less pigmentation on the ventral side (Figure 1i,k).

Amelanistic (AM) individuals of *M. auratus*, a common cultivated breed, lack all black and brown pigmentation (Figure 1c,d), both in the skin (Figure 1f,h) and in the iris and retina (Figure 1j,l). Despite the lack of melanic pigmentation, we still find hints of the stripe pattern. In females, both dls and mls can be identified as yellow longitudinal bands flanked by white coloration (Figure 1c,f). Dominant AM males are almost entirely white (Figure 1d,h), with a slight silvery and bluish appearance, both in the skin and in the iris (Figure 1h,l).

In an effort to better understand the genomic basis of this amelanism in this species, we sequenced the genomes of a *M. auratus* WT and AM individual at 49.4 and 24.9× coverage, respectively. The inheritance of the trait suggested a Mendelian recessive basis, as AMs produced only AMs and some of the WT pairs produced clutches of WT individuals with a few AM individuals (quantification see below). To screen for putatively causal mutations, we first screened for homozygote, AM-specific non- and missense mutations that could drive the loss of melanic pigmentation in AMs (Tables S1 and S2; detailed methods described in Data S1). Because major effect genes controlling melanophore differentiation and melanin synthesis have been comprehensively described (Braasch, Schartl, & Volff, 2007) and have highly conserved functions across vertebrates (Fukamachi et al., 2004; Miller et al., 2007), we focused on mutations that were located in known coloration genes (Figure 2a). We found non-synonymous mutations in three pigmentation genes including phosphoribosylaminoimidazole carboxylase (*paics*), leukocyte receptor tyrosine kinase (*ltk*), and the oculocutaneous albinism type 2 gene (*oca2*). Yet, all variable sites showed also variation among closely related cichlid species (*paics:* T in *Metriaclima zebra*, C in *Astatotilapia calliptera; ltk:* C in *Metriaclima zebra*, T in *Astatotilapia calliptera; oca2:* T in *Metriaclima zebra*, C in *Pundamilia nyererei*), suggesting that they are commonly variable within populations and between species and are not causally involved in the amelanism.

Another possibility is that this phenotype is caused by a larger deletion that results in gene or exon loss of a coloration gene. To screen for such deletions, we compared the coverage of all exonic sequences across the genome (Tables S3 and S4; detailed methods described in Data S1). Only in one known pigmentation gene did we find such a deletion. The gene *oca2* lacked the second exon (relative coverage in WT: 0.9×; AM: 0×). This deletion covered a total of 5.4 kb including exon 2 and parts of the flanking introns 1 and 2 (Figure 2b). To confirm the association of the deletion with amelanism, we designed primers for the deleted region (exon 2 specific primers, and primers flanking the deletion; Figure 2c). Genotypes of 41 additional individuals (14 amelanistic individuals) from the same breeding pool perfectly associated with the phenotype (complete penetrance; Fisher's exact test: $p = 2.8 \times 10^{-11}$), with all WT individuals being positive, and all AM individuals being negative for the amplicon encompassed in the 5.4 kb region (Figure 2d). Phenotyping distribution of the offspring of heterozygous individuals is also in accordance with the expected 1:3 ratio of a recessive Mendelian trait (two clutches, 4 AM: 17 WT; $\chi^2 = 0.4$, $df = 1$, $p = 0.53$).

Significance

Genomic approaches are a powerful tool to uncover the genetic basis of pigmentation phenotypes. Genome sequencing of the Malawi golden cichlid fish (*Melanochromis auratus*) and an amelanistic morph of the species identifies the loss of the second exon of *oculocutaneous albinism II* (*oca2*) in amelanistic individuals, a gene that is considered the most common cause of albinism in humans. Exon 2 contains a conserved motif previously suggested to be crucial for Oca2 delivery to melanosomes. Our work confirms the functional importance of this motif within the extracellular domain of Oca2 and supports the striking and repeated involvement of *oca2* variants in amelanism and albinism across vertebrates.

Next, we tested whether the deletion resulted in a truncated mRNA or alternative splicing event resulting in a fusion of exon 1 and exon 3. Therefore, we performed PCRs on cDNA extracted from the eyes of WT and AM individuals using a forward primer in exon 1 and a reverse primer in exon 6 (detailed methods described in Data S1). AM individuals had a ~100-bp shorter band corresponding to the 96-bp deletion of exon 2 (Figure 2e). Heterozygote individuals (that showed the $oca^{WT/WT}$ phenotype) had as expected both the normal band (oca^{WT} allele) and the band lacking exon 2 (oca^{AM} allele; Figure 2e). The second exon of *oca2* contains a previously described highly conserved acidic di-leucine motif (Figure 2f; Sitaram et al., 2012, 2009).

In summary, genome resequencing of amelanistic and wild-type individuals of *M. auratus* reveals a deletion of the second exon of *oca2* as the likely cause of the amelanism. In the past, *oca2* has been associated with albinism in humans and mice (Brilliant, Gondo, & Eicher, 1991; Rinchik et al., 1993). In fact, in humans it is the most common cause of albinism and hypopigmentation (oculocutaneous albinism type 2; Manga, 2018). More recent findings support a similar and very conserved function across all vertebrates including reptiles and teleost fishes. For example, CRISPR-Cas9 mutants of *oca2* in the Mexican tetra, *Astyanax mexicanus* (Klaassen, Wang, Adamski, Rohner, & Kowalko, 2018), spontaneous mutations in the Medaka, *Oryzias latipes* (Fukamachi et al., 2004), or a truncation of the *oca2* transcript in the corn snake, *Pantherophis guttatus* (Saenko et al., 2015), result in similar amelanistic phenotypes. The precise function and subcellular localization of Oca2 is still debated (Sitaram et al., 2009). Oca2 is a 12-transmembrane domain protein that localizes to melanosomes where it is thought to be involved in the maturation and trafficking of tyrosinase, one of the key enzymes of melanin synthesis (Manga, 2018; Sitaram et al., 2009). Furthermore, it has been proposed to regulate tyrosinase activity via regulation of melanosome pH (Bellono, Escobar, Lefkovith, Marks, & Oancea, 2014). Exon 2, which is deleted in amelanistic individuals of *M. auratus*, translates into parts of the cytoplasmic N-terminal domain that is crucial for trafficking of the protein

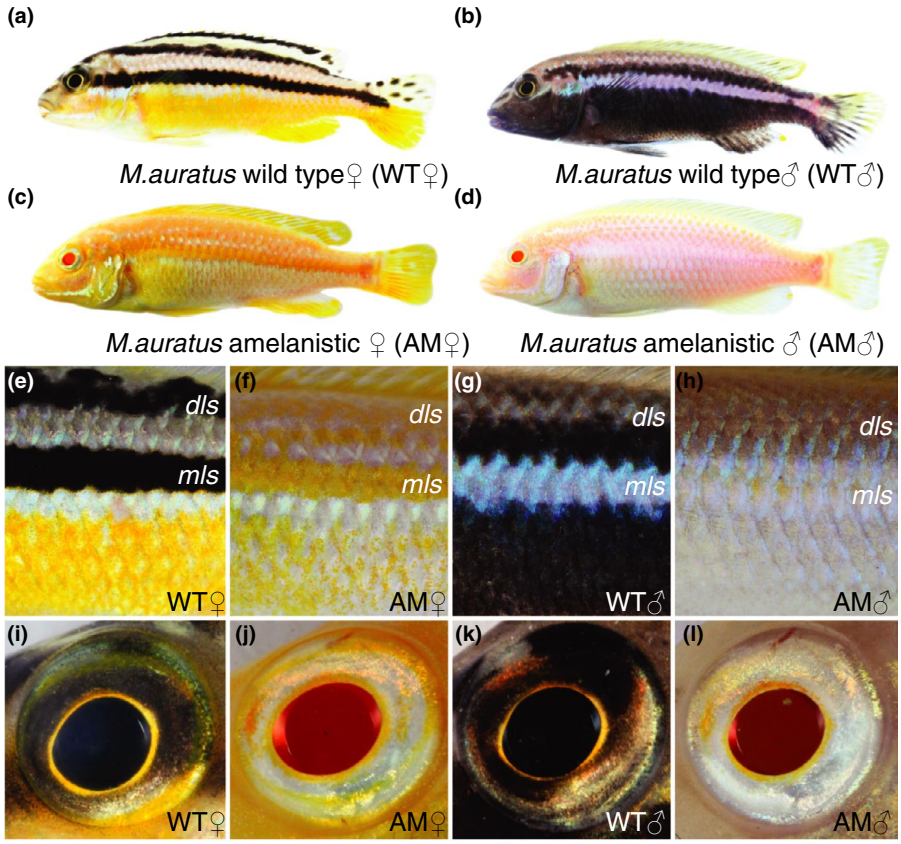

FIGURE 1 The coloration phenotype of *Melanochromis auratus* morphs. (a) *M. auratus*, wild-type (WT) female. (b) *M. auratus*, dominant WT male. (c) Amelanistic (AM) female of *M. auratus*. (d) Dominant AM male of *M. auratus*. (e) Skin of a female WT showing the characteristic stripe pattern. (f) AM individuals show a similar distribution of yellow and white pigmentation but lack the melanic stripe patterns of WT individuals in the dorsolateral (dls) and midlateral stripe (mls) region. (g) Dominant males are mostly brown to black, with a fine dls and a thicker mls with white to blue iridescent color. (h) Dominant amelanistic males lack the dark pigmentation and are white to flesh-colored, while largely lacking yellow or orange pigmentation. (i) The eye of a female WT with the darkly pigmented iris and a yellow ring around the lens. The ventral side of the iris is less pigmented. The dark-pigmented retina can be seen through the lens. (j) The eye of an AM female, lacking dark pigments, but showing yellow and iridescent pigmentation. The non-pigmented retina is shining through. (k) The eye of a male WT with similar but darker pigmentation than the WT female. (l) AM male eye with less yellow pigmentation than AM females

from endosomes toward maturing melanosomes (Sitaram et al., 2012, 2009). Moreover, the exon contains an acidic di-leucine motif that is a crucial signal for melanosome delivery (Figure 2f). Therefore, it can safely be assumed that Oca2 is not able to localize normally to melanosomes, thereby blocking the melanin synthesis pathway, which in turn results in the observed amelanistic phenotype. This finding is also supported by the strong conservation of the complex molecular mechanisms involved in Oca2 trafficking to melanosomes as it has been previously suggested (Sitaram et al., 2012, 2009).

It is interesting that indications of the typical color patterns of *M. auratus* can still be found in amelanistic individuals, including the white delineation of the mls in females, the reduction in yellow/orange pigmentation in males (Figure 1f), and the more widespread iridescent coloration in males (Figure 1h). As these patterns are usually generated and maintained through interaction between different pigment cells including melanophores, xanthophores, and iridophores (Patterson & Parichy, 2013), this suggests that melanophores might be still present and still, although now lacking melanin, contribute to shaping of the conserved pigmentation pattern. This hypothesis is also supported by the maintained expression of *oca2* as well as similar observations in the corn snake, *P. guttatus* (Saenko et al., 2015).

Because cichlids are increasingly used for studying developmental mechanisms (Bloomquist, Fowler, Sylvester, Miro, & Streelman, 2017; Woltering, Holzem, Schneider, Nanos, & Meyer, 2018), also using transgenic reporter lines (Juntti, Hu, & Fernald, 2013; Kratochwil, Sefton, Liang, & Meyer, 2017), non-melanic mutant lines are also a helpful resource as they improve signal detection for in vivo imaging and staining methods that are aggravated by melanic pigmentation. Amelanistic *M. auratus* might be therefore a useful system for studying cichlid pigment pattern formation as well as the sexual dimorphisms of *M. auratus* (Figure 1).

Through genome resequencing, we identified the likely causal genetic basis of amelanism in *M. auratus*: the lack of the second exon of *oca2*, which contains a crucial motif for trafficking to melanosomes and thereby results in a non-functional Oca2 protein. This

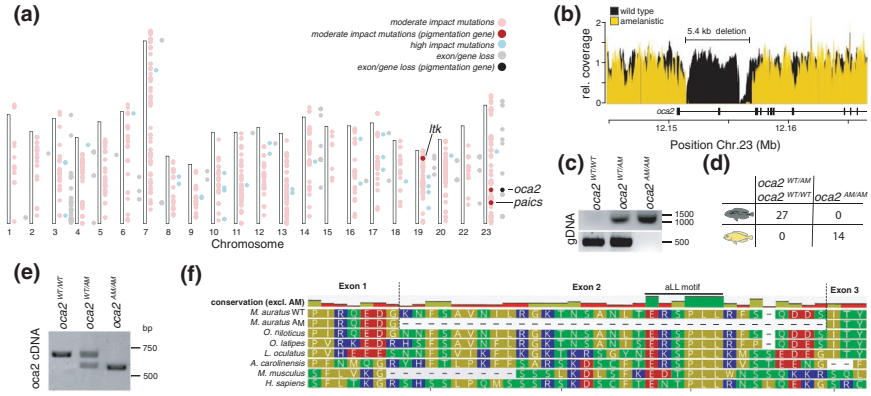

FIGURE 2 The genomic basis of amelanism in *Melanochromis auratus*. (a) Genome-wide screen for non-/missense mutations and exon/gene loss reveals three coloration genes with non-synonymous mutations (that are however variable among cichlids) and one coloration gene, *oca2*, with exon loss. (b) Relative sequencing coverage of AM (yellow) and WT (black) individuals at the *oca2* locus identifies a 5.4-kB deletion including exon 2 of *oca2*. (c) PCR validation of the deletion using primers specific for exon 2 sequence (bottom; only amplifying in $oca2^{WT/WT}$ and $oca2^{WT/AM}$) and specific for the deletion (bottom; only amplifying in $oca2^{AM/AM}$ and $oca2^{WT/AM}$). (d) Amelanism is a recessive Mendelian trait. All individuals homozygous for the deletion of exon 2 $oca2^{AM/AM}$ are amelanistic, while $oca2^{WT/AM}$ and $oca2^{WT/WT}$ individuals have normal coloration. (e) PCR on cDNA confirms the exon loss and ligation of *oca2* exon 1 and 3 in AM individuals. (f) Alignment of the amino acid sequence translated from exon 2 shows the loss of a highly conserved acidic di-leucine motif in that constitutes the likely cause of the amelanism of *M. auratus*

species of cichlid fish is a suitable model for the study of pigment pattern formation as well as for developmental research adding an ecologically and evolutionarily well-established species to the two classical model teleost species zebrafish and medaka.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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