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Identification and Characterization of Gene Expression Involved in the Coloration of Cichlid Fish Using Microarray and qRT-PCR Approaches

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Abstract It has been suggested that speciation on the basis of sexual selection is an important mechanism for the generation of new species for East African cichlids, where male body coloration is one of the major discriminatory factors used by females in mate choice. To gain insight into the molecular basis of cichlid coloration, we studied the Lake Malawi cichlid *Pseudotropheus saulosi*, comparing transcription in the bright blue skin of males to the yellow skin of females. Our cDNA microarray experiments identified 46 clones that exhibited expression differences between the two sexes, of which five were confirmed to be differentially expressed by relative quantitative real-time PCR (qRT-PCR). This gene list includes a representative from the endosomal-to-Golgi vesicle trafficking pathway, *Coatomer protein complex*, *subunit zeta-1* (*Copz-1*), which is known to be a critical determinant of pigmentation in humans and zebrafish. With the support of microscopic images of the skin of these specimens, we interpret the transcriptional differences between the blue males and yellow females. Here, we provide insight into the putative functional diversification of genes involved in the coloration of cichlids and by extension, on the evolution of coloration in teleost fish.

Keywords Adaptive evolution · Cichlid species flocks · Sexual selection · *Copz* · *Collagen 1 alpha*

Introduction

The family Cichlidae is composed of about 3,000 species, making it one of the most species-rich families of vertebrates. More than 1,500 endemic species alone live in the Great Lakes of Eastern Africa. Because of their impressive phenotypic diversity, their extremely fast speciation rates, and their unique degree of endemism, cichlid fishes have attracted the attention of researchers from several biological sub-disciplines. African cichlids have become a prime model system for the study of evolutionary processes, especially for explosive rates of speciation, the formation of adaptive radiations and a multitude of mechanisms of speciation (Fryer and Iles 1972; Meyer 1993; Kocher 2004; Salzburger and Meyer 2004; Elmer et al. 2009a, b).

The evolutionary success of the East African cichlids has been attributed to a combination of ecological opportunities (after the colonization of large lakes), and behavioral (maternal mouthbrooding), and morphological innovations (egg-spots, color polymorphisms, and pronounced sexual dichromatism) (Fryer and Iles 1972; Meyer 1993; Galis and Metz 1998; Kocher 2004; Salzburger et al. 2005; Elmer

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et al. 2009b). Sexual selection has been proposed as an important mechanism in their speciation. This has been demonstrated by field observations (van Oppen et al. 1998; Salzburger et al. 2006; Elmer et al. 2009b) and complementary mate choice experiments in the laboratory (Barlow et al. 1977, 1990; Knight et al. 1998; Seehausen et al. 1999). More specifically, ecological evidence that sexual selection plays a role in speciation came from the inferred breakdown of visual reproductive barriers under monochromatic light conditions or in turbid waters (Seehausen et al. 1997; Seehausen et al. 2008). Recent research on visual pigments has added to these observations and suggests that the way in which cichlids perceive the world visually changes rapidly throughout evolution and development (Carelton et al. 2008; Hofmann et al. 2009) and ultimately modifies their ability to discriminate potential mates. Taken together, these results suggest that male body hue is one of the primary discriminatory factors among a hierarchy of visual and probably olfactory cues used by females.

In cichlids, it has been shown that species with highly similar colors have evolved repeatedly within and among lakes, with a predominance of blue and yellow colors (Meyer 1993; Stiassny and Meyer 1999; Seehausen et al. 1999; Seehausen and van Alphen 1999; Allender et al. 2003; Elmer et al. 2010). This has resulted in astonishing phenotypic diversity despite their genetic uniformity due to their extremely young age (Meyer et al. 1990; Sturmbauer and Meyer 1992; Allender et al. 2003; Verheyen et al. 2003). Furthermore, body color differences evolved intraspecifically and a rich palette of color polymorphisms is known from cichlid assemblages in the New as well as the Old World. These color morphs can be so strikingly different that the casual observer would assign them to different species (Fryer and Iles 1972). Finally, many cichlid species, and in particular the modern haplochromine cichlids, are sexually dichromatic, with the male being the colorful sex and females typically having a dull cryptic body color (Salzburger et al. 2005).

Coloration in vertebrates is based on the number and distribution of three types of pigment cells (chromatophores): dark melanophores, yellow to orange xanthophores, and reflective iridophores, responsible for a white or silvery coloration (Bagnara and Hadley 1973; Fujii 1993a, 2000; Mellgren and Johnson 2002; Braasch et al. 2008). In teleost fishes, at least two more classes of chromatophores have been identified: the white leucophores and the blue cyanophores (Bagnara 1998). The chromatophores originate from neural crest cells that migrate in the ectoderm of the embryo during early ontogeny (Bagnara et al. 1979).

Each chromatophore has a characteristic set of pigments that reside in special pigmentary organelles (Fujii 1993a, b; Bagnara 1998). These organelles are described as melanosomes, which contain melanin in melanocytes (Charles and Ingram 1959; Drochmans 1960; Birbeck 1963); pterinosomes, which contain pteridines in xanthophores (Matsumoto 1965a; Kamei-Takeuchi and Hama 1971; Bagnara 1976); and the reflective platelets, which contain purines in iridophores (Bagnara and Stackhouse 1961; Bagnara et al. 1979). Pterinosomes and melanosomes are derived from the Golgi complex (Obika 1993). They contain a species-specific set of pteridines that appear at the same ontogenetic stage as when xanthophores undergo differentiation (Matsumoto et al. 1960; Hama 1963; Obika 1963; Matsumoto 1965b). Sepiapterins, drosopterins, and several colorless pteridines can be detected as yellow pigmentation that first becomes visible within pterinosomes (Obika 1963; Kamei-Takeuchi and Hama 1971).

Determining the genetic mechanisms that drive the evolution of cichlid color patterns will aid in the understanding of their explosive speciation. As cichlids experienced the fish-specific genome duplication the increased number of genes, followed by lineage-specific gene loss, sub-functionalization and regulatory evolution might be a genomic explanation for their species richness (Taylor et al. 2001a, b; Santini et al. 2003; Braasch et al. 2006, 2007, 2008, 2009a, b, c). The utility of the cichlid genome has increased in recent years and is coordinated by the cichlid genome consortium (http://cichlid.umd.edu/CGC index.html), which has developed genetic linkage maps (Kocher et al. 1998; Albertson et al. 2003; Sanetra et al. 2009), BAC libraries (Katagiri et al. 2001; Watanabe et al. 2003; Lang et al. 2006), cDNA and EST libraries (Watanabe et al. 2004; Salzburger et al. 2008), and microarrays (Renn et al. 2004). Genomic tools have previously been used to successfully identify the genetic basis of sex-biased coloration in cichlid fishes. For example, QTL analysis has identified that the genetic basis of the orange blotch color pattern in Lake Malawi cichlid fishes is a mutation in the cis-regulatory region of the *Pax7* gene, which results in a significant increase in expression of this gene (Streelmann et al. 2003; Roberts et al. 2009).

It stands to reason that other regulatory changes underlying cichlid color polymorphisms might be rapidly identified using genome-wide transcription-profiling techniques such as DNA microarray. DNA microarray technology permits genome-wide comparisons of gene expression in different tissues or developmental stages, in healthy or diseased individuals, and in response to different environmental conditions (see, e.g., Crawford et al. 1999; Gasch et al. 2000; Michaut et al. 2003; Segal et al. 2003; Whitehead and Crawford 2005; Abzhanov et al. 2006). This is achieved through the hybridization of labeled RNAs to slides that have been "spotted" with cDNA clones from a given organism. The relative transcription can be calculated for given samples, through comparing the intensity of

labeling at any given "spot." Here, we investigated the genetic basis of color polymorphism in a cichlid species using an approach that combines microarray and relative quantitative real-time PCR (qRT-PCR) with morphological investigations.

We examined the transcriptional basis of sexually dimorphic coloration in the Lake Malawi cichlid *Pseudotropheus saulosi* (PS). Our examination compared the expression of ~6000 clones from the blue skin of a PS male (Fig. 1a) to the yellow skin of a PS female (Fig. 1b), using a cDNA microarray chip. This chip was constructed using sequenced ESTs from the "pinky" library, derived from a range of tissues including the skin of the African cichlid *Astatotilapia burtoni* (AB), detailed in Salzburger et al. (2008). AB and PS are closely related and it has been shown that heterologous microarray hybridization can yield biologically meaningful data even in much more distantly related species (Renn et al. 2004; Cummings et al. 2008). After identifying the genes that showed a difference in expression between skin samples of the different color morphs of PS, we used qRT-PCR to confirm and expand upon the results of the microarray experiments. We also analyzed the expression of the previously isolated color genes *csf1ra* and *csf1rb*, as *csf1ra* is important to xanthophore cell fate and is known to be expressed in the yellow egg dummies of African cichlids (Williams et al. 2002a, b; Parichy and Turner 2003; Braasch et al. 2006; Salzburger et al. 2007).

Materials and Methods

Tissue Samples

A single brood of PS was reared in a single tank, under standard conditions (12 h light; 12 h dark, 25°C water temperature). Adult fish were killed after anesthetizing them with Tricaine (MS222; Sigma) and were frozen in liquid nitrogen and stored at −80°C. Colored skin pieces of 1.0 cm × 0.5 cm were later removed from the bright blue (males) and yellow (females) (Fig. 1a, b; Supplementary Fig. S1) from regions adjacent to one of the dark bands on the central part of the fish's flank. Scales were left on the skin samples because their removal destroys most of the chromatophores (data not shown). We used fluorescent microscopy methods to detect xanthophores in these samples (see Odenthal et al. 1996). The skin was mounted in methyl cellulose with 1 drop of 0.2% 3-aminobenzoic acid ethyl ester (Sigma) and 1 drop of dilute ammonia, 0.1% β-mercaptoethanol, pH 10.0. Pictures were taken under transmitted light and UV light (DAPI-filter) with an Axiophot 2 microscope (Zeiss). The dilute ammonia liberates pteridines from their protein carriers at a high pH. These are then visualized as light green fluorescence (Epperlein and Claviez 1982; Odenthal et al. 1996).

RNA was purified from skin samples from the flanks of 10 males and 9 females of identical sizes and locations to those used for the histology analysis (Supplementary Fig. S1). Total RNA was purified using TRIZOL LS (Life Technologies) according to the manufacturer's instructions. Purified RNA was resuspended in RNase-free water. The concentration of total RNA was calculated with an Eppendorf Biophotometer 6131, and the quality was assessed through agarose gel electrophoresis. First strand cDNA was synthesized from 5 µg of total RNA of each sample, using Superscript III Reverse Transcriptase (Invitrogen), primed by oligo d(T). To test for genomic DNA contamination, cDNA was used for PCR amplifications of *gapdh* and *actin* genes that spanned at least one intron (see Supplementary Table S1).

Hybridization Experiments

Here, we used a microarray generated from a normalized cDNA library derived from whole "pinky" juveniles of the African cichlid *Astatotilapia burtoni* (Salzburger et al. 2008). Scienion (Berlin, Germany) performed the spotting; where 1–3 ng of each clone was applied to the chips, in addition to a 96-well "control plate." In total, the array contained 6,144 spots from the *A. burtoni* EST library, with an average length of 615 bp. The control plate contained fragments of 12 candidate "color" genes that were isolated from cDNA of *A. burtoni*, in duplicate and water blanks. The candidate color genes include *pdgf1ba*, *pdgf1bb*, *csf1ra*, *csf1rb*, *endrb1*, *wnt1*, *kita*, *aim1*, *mc1r*, *sox10b*, from previously published sequences (Braasch et al. 2006; Lang et al. 2006; Salzburger et al. 2007), plus *pax3*. Hybridization probes were generated from pooled cDNA synthesized from a total of 15 µg of total RNA extracted from three different individuals for each sex. The hybridization probes were labeled using the Labelstar Array kit (Qiagen) with FluoroLink Cy 3-dCTP and Cy 5-dCTP (Amersham Biosciences) according to the manufacturer's protocol. The labeled cDNAs were hybridized to the chips at 60°C for 16–21 h, washed with SSC buffer, and read with a Genepix 4000B microarray reader (Axon Instruments). Three arrays (an array used later as reference array and two dye-flip replicates of it) were used to examine all spotted cDNAs as uneven background fluorescence obscured some of the spots. The dye-flip (also known as dye swap or reverse labeling) technique generates paired slides where, on the first slide, one mRNA sample is labeled by Cy5 and the other mRNA sample is labeled by Cy3, while, on the second slide, the labels for the two samples are exchanged. This technique removes dye-specific effects (Sartor et al. 2003; Yongxiang Fang et al. 2003).

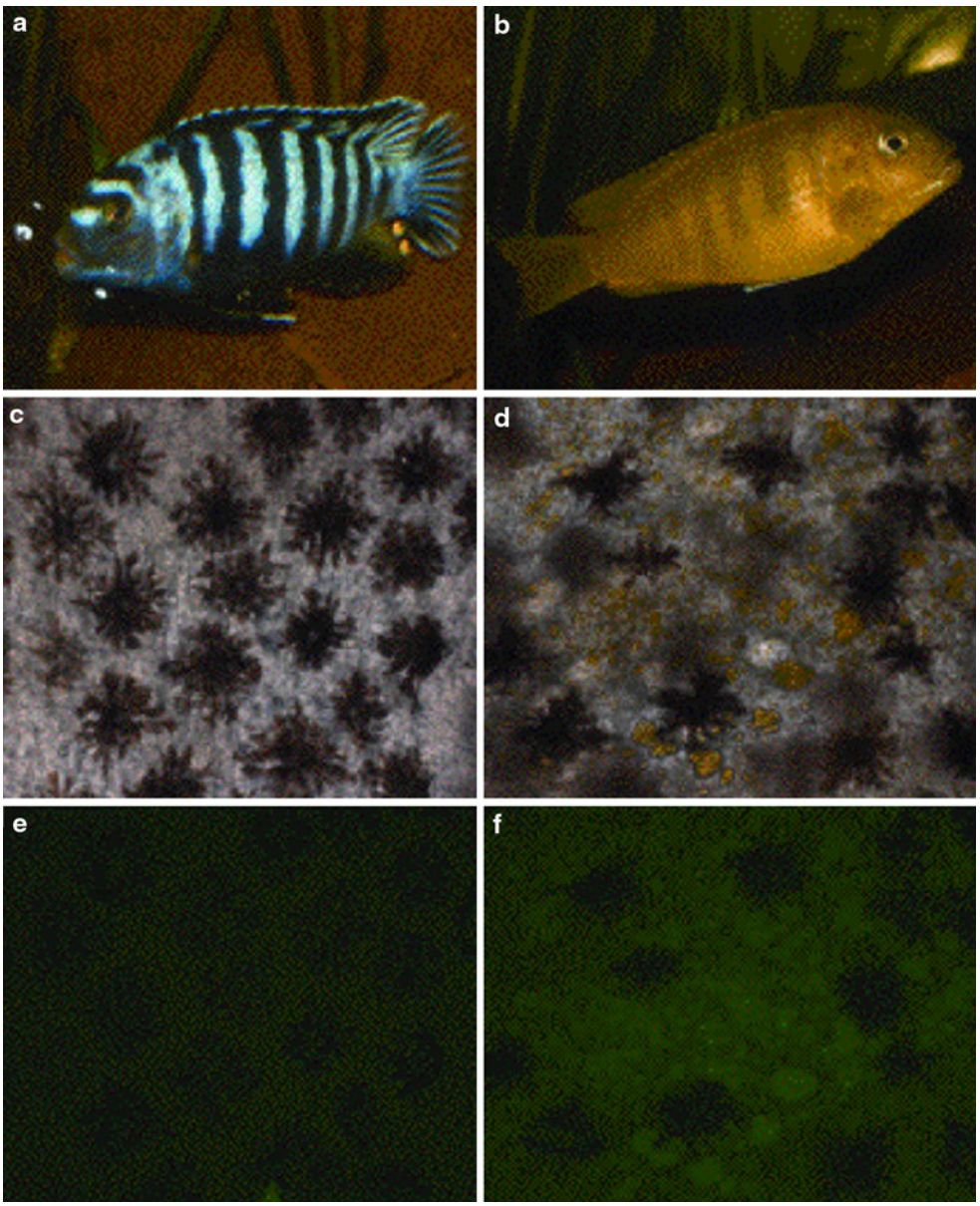

Fig. 1 Images of fish and their skin under transmitted light and UV light. **a** PS male, **b** PS female, **c** skin of PS male under transmitted light, **d** skin of PS female under transmitted light, **e** skin of PS male under UV light, and **f** skin of PS female under UV light

Analysis

After normalization (performed with the Genepix Pro 4.0 software on the basis of the mean of the intensities ratios), 2,196 of the spots showed a difference in expression in at least one dye-flip experiment. From this list, we identified the spots that showed a difference in their log ratios of intensities that was above 2-fold, between the reference array and at least one of the dye-flip experiments. The reliability of the genes in the microarray list was tested through qRT-PCR. Additionally, we analyzed the expression of *csf1ra* and *csf1rb*, as *csf1ra* is known to be strongly expressed during the specification of yellow egg spots in cichlid fishes (Salzburger et al. 2007).

qRT-PCR Experiments

For the selected 46 clones, sequences were obtained using the original insert from the cDNA library amplified with primers SP6 and T7 (see Supplementary Table S1) using an ABI PRISM 3100 sequencer (Applied Biosystems). Of the 46 clones, all contained unique sequences except for 04C E05 and 14C E03, as determined through alignment with Sequencher 4.0 (10% minimum overlap), thus expression analysis of this gene (parvalbumin beta) is

based on primers designed to 04C E05 only. Specific primers for each unique clone and for *csf1ra* and *csf1rb* (see Supplementary Table S1) were then designed using Primer Express (Applied Biosystems).

For the qRT-PCR experiment, fresh RNA was extracted from the skin of four male and four female PS (see Supplementary Fig. S1). Extractions were performed using Trizol (Invitrogen), with subsequent reprecipitation with 2 M LiCl, then 0.3 M NaOAc and 2 vol EtOH (Sambrook and Russell 2001). The purity of our RNA was determined to be of a high level, as A260/A280 values were between 1.9 and 2.1. For synthesis of cDNA, 2.0 μg of RNA was transcribed using the Qiagen Quantitect cDNA system, which includes an initial gDNA removal step. After gDNA removal, RNA concentration was calculated for each sample using a RiboGreen Assay, which enables accurate comparison of qRT-PCR samples without using a housekeeping gene for normalization (see below).

qRT-PCR experiments were performed with the iQ SYBR Green Supermix (BioRAD) with between 1.5 and 6.0 pmol of each primer. Amplification and detection of products was performed using the CFX96 Real Time System (BioRAD). The following thermoprofile was used for all qRT-PCR primer pairs (see Supplementary Table S1): 95°C for 10 s for 1 cycle; then 95°C for 5 s, 60°C for 15 s, 72°C for 20 s, for 44 cycles; then a melt curve from 60 to 95°C with 0.5°C increments for 5 s each.

Relative expression was calculated without a housekeeping gene comparison. This technique was used because we found that normalization to *β-actin* and *gapdh* gave conflicting results (data not shown). We employed the methods described in Cummings et al. (2008), using the formula $1/E^{\text{avgCT}}$, where $E = 10^{-(\text{slope})}$, as described in Simon (2003). Results were scaled against the RNA quantity calculated using the RiboGreen Assay and were compared using an independent *t* test. We used a Shapiro–Wilk test for normality of our expression profiles (Shapiro and Wilk 1965) and found that all of the clones with significant *t* test results were included in this list.

We obtained additional 3′-sequence of the clones that showed statistically significant expression difference using the forward primer designed for qRT-PCR or newly designed internal primers when the insert was long. These sequences were blasted in NCBI (using blastx, or tblastx and blastn when blastx did not deliver a significant result) on 16.08.09, and gene ontologies were searched using different databases and literature sources.

Results

When male skin was examined under transmitted light (Fig. 1c), we observed melanophores with melanosomes dispersed in the cells. Fewer melanophores were observed in the skin of the female of PS (Fig. 1d), but these looked very similar to the ones present in the males' skin. Furthermore, pterinosomes were also observed as yellow vacuoles under transmitted light, however, the whole xanthosome remained difficult to identify. No fluorescence was observed in the male's skin under UV light (Fig. 1e), suggesting that no or very few xanthophores are present in males. In the females' skin, we observed bright stains that autofluoresced against background under UV light (Fig. 1f). Some of these stains show the same pattern as the yellow vacuoles (Fig. 1d), but overall we observed many more of these stains in the picture under UV light than of yellow vacuoles (Fig. 1f).

In our analyses of the microarray experiments, we selected clones that displayed a greater than 2-fold difference in terms of log ratios between the reference array and at least one of the two dye-flip arrays. We distinguished the selected clones, grouping them into three categories depending on the robustness of the data of the second dye-flip array. When the data showed a difference between log ratios of the intensities greater than 1.5-fold in both arrays, genes were grouped in category A. In the reverse case, i.e., if the difference between log ratios of the intensities of the second array was less than 1.5-fold, clones were included in category B. Finally, category C contains genes selected in the absence of data for the second dye-flip experiment due to the presence of background dye on a particular spot.

We identified 46 clones, representing 45 unique sequences and tested their expression profiles with qRT-PCR in addition to *csf1ra* and *csf1rb*. None of the control skin genes spotted onto the chip were included in this list. Of the 47 genes, 40 were successfully amplified and 5 were confirmed to display statistically significant expression differences in qRT-PCR experiments in PS (Table 1; Fig. 2). The one gene that could not be amplified was too short for satisfactory primers to be designed and was thus omitted from the analysis (21A E10). We found a relatively low concurrence between microarray and qRT-PCR results, which is likely to relate to an initially high false positive discovery rate in the microarray analysis and the use of new biological replicates in the qRT-PCR, as they were collected on a separate occasion compared to the pooled samples used in the microarray (see "Discussion" section). For some genes, we also found a very high level of variability between these biological replicates in the qRT-PCR (Supplementary Fig. S2), which would have been obscured by the pooled samples used in the microarray.

After additional sequencing, the genes could be further characterized by blast searches (Table 1). Initially, we used the blastx algorithm to query the nr/nt nucleotide collection, and in the case that there was no significant hit, blastn

Table 1 Clones selected after normalization of the microarrays and their top hits from the Genbank database, as determined through blastx, blastn, or tblastx

Clone	Microarray log intensity values			qRT-PCR	BLAST identification		
	Reference	Dye-flip a	Dye-flip b	*P* value	ID	Accession	E value
Category A							
02A F10	−3.444	0.314	−2.687	0.408	Defender against cell death 1 (DAD1)	ABD79023	4.00E−55
02B A11	−4.092	0.770	−2.847	0.784	Annexin max 3	NP_001098295	9.00E−90
03A A11	3.929	0.603	3.395	0.425	No significant similarity		
03A B02	−2.797	0.567	−1.841	0.889	60S ribosomal protein L5	ACI66198	8.00E−115
03A G01	−1.793	1.232	−2.872	0.012*	Type 1 collagen alpha 2	BAD77969	1.00E−144
03B B02	−2.381	0.084	−1.780	0.798	40S ribosomal protein S11	ACO15454	3.00E−81
04A F07	1.187	−0.702	3.237	0.279	Parvalbumin	ACF23534	8.00E−15
04C E05	−2.626	0.541	−1.172	0.268	Parvalbumin	ADA70320	3.00E−44
11A G01	−2.685	0.504	−2.517	0.376	Heat shock protein 90 beta	BAF92790	5.00E−92
11B A05	1.827	−0.216	2.131	0.161	Leucine zipper-EF-hand containing protein	XP_001251731	3.00E−13
11B A10	−3.121	−0.036	−1.774	0.360	40S ribosomal protein SA	ACO09157	4.00E−115
12B B04	−3.767	−0.519	−36.175	0.741	Major histocompatibility complex II	CAP47207	1.00E−19
12B E12	−2.729	0.483	−1.971	0.448	Ribosomal protein L19	CAD91441	7.00E−51
12C D12	−2.670	0.535	−2.022	0.746	No significant similarity		
13B D03	−2.561	0.670	−2.979	0.246	No significant similarity		
13D H05	−1.564	1.222	−1.507	0.557	Beta hemoglobin A	Q9PVM2	6.00E−67
14D D11	−1.171	1.102	−2.159	0.073	Similar to Ribosomal protein L29	XP_001151583	8.00E−24
18B H11	−1.835	0.279	−2.903	0.145	No significant similarity		
csf1ra				0.261			
csf1rb				0.888			
Category B							
01A G09	−2.354	0.169	−1.404	0.980	Ribosomal protein S14	CAA69615	3.00E−73
01B B11	−2.951	−0.161	−0.519	0.277	Myosin light chain 2	AAX34414	3.00E−04
01C G09	−1.793	0.321	−1.285	0.551	Beta actin	AB037865	0
03B B07	−2.027	0.819	−1.404	0.808	Ubiquitin/ribosomal fusion protein	CAJ90900	9.00E−64
03C F01	−2.252	0.291	−1.221	0.413	40S ribosomal protein S17	ACN09866	4.00E−67
03C H09	−1.706	0.998	−1.001	0.257	Cold-shock domain protein	DQ840135	0
03D A07	−1.521	0.967	−0.490	0.772	60S ribosomal protein L7a	ACQ59023	9.00E−21
11B E02	−1.637	0.631	−0.950	0.291	Ribosomal protein S2	BAF45464	2.00E−77
12A H03	−1.834	1.439	−1.406	0.515	Thymosin beta-4 putative	BT082182	1.00E−87
12D F03	−1.678	0.890	−0.827	0.492	Ribosomal protein L13a	BAF98661	1.00E−90
13C E01	0.237	0.155	2.443	0.142	Fast/white muscle troponin T	ABF20456	1.00E−41
14C_E03	−0.396	0.742	1.903		Parvalbumin 2	ADA70320	2.00E−44
14C H09	−1.080	−1.102	−2.476	0.045*	Coatomer subunit zeta-1	ACQ58956	2.00E−83
15A B05	−1.112	−0.422	−2.451	0.502	Collagen, type X, alpha 1	ADG29155	2.00E−68
15A D07	−1.196	−0.458	−21.025	0.211	No significant match		
17A E10	1.019	−0.230	2.139	0.038*	Tetraodon nigroviridis full-length cDNA	CR633442	8.00E−123
19A D07	−1.787	0.255	−0.402	0.947			
Category C							
03C D08	1.194	x	−2.514	0.084	No significant similarity		
13C B02	1.096	x	−1.869	0.976	LINE partial sequence, clone:C8	AB017056	7.00E−14
15B H02	−1.387	x	1.247	0.183	Adenylate kinase	CAG12406	2.00E−23
15D G03	−1.000	x	1.236	0.123	RAB11B, member RAS oncogene family	NP_001012569	5.00E−117
15D G09	−1.548	x	1.231	0.039*	No significant similarity		
16D B11	−1.452	x	1.026	0.041*	No significant similarity		

Table 1 continued

Clone	Microarray log intensity values			qRT-PCR	BLAST identification		
	Reference	Dye-flip a	Dye-flip b	*P* value	ID	Accession	E value
16D D05	−1.444	x	1.308	0.198	No significant similarity		
19B E11	−2.352	0.809	x	0.269	Elongation factor 1 a	BAB83860	3.00E−85
19C D03	1.080	x	−1.503	0.265	Calsequestrin 1	AB442012	3.00E−139
20C F08	−1.936	x	2.032	0.121	No significant similarity		

*$P < 0.05$

and tblastx were used. For those with blast hits smaller than $1e^{-15}$, the name of the best hit will be used as an identifier in the remainder of the manuscript. We refer to genes as "similar to" when sequence similarities were greater than $1e^{-5}$ (Altschul et al. 1990). The list of five statistically significant genes includes *Collagen 1 alpha*, *Coatomer protein complex*, *subunit*, *zeta-1* (*Copz-1*), one clone with a hit to the 3′-UTR of a ubiquitin-conjugating enzyme, and two clones without any annotation in the Genbank database (17A E10 and 16D B11). Both unannotated clones show strong hits to other cichlids in the BLAST EST database, and 17A E10 has significant hits to ESTs from other fish species. The clone 16D B11 had only a weak hit to the gilthead sea bream EST collection (*Dicentrarchus labrax*; E value = 0.005).

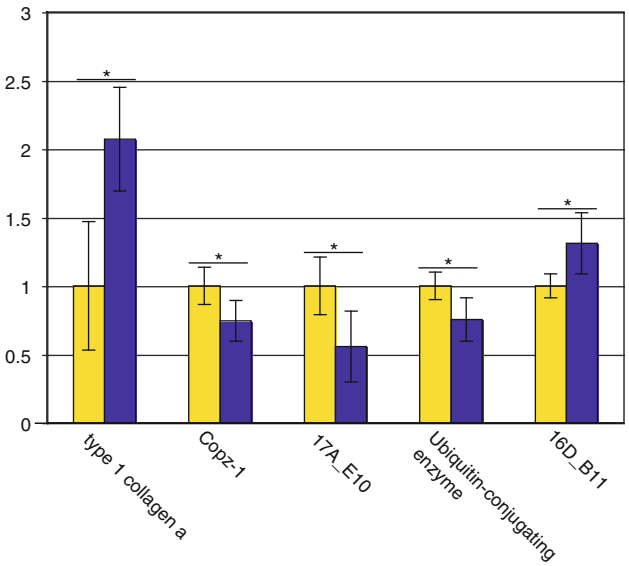

Fig. 2 Genes that differ in expression between males and females, as confirmed through qRT-PCR. The relative expression values are scaled so that female expression is equal to 1. *Yellow bars* represent PS female expression and *blue bars* represent the PS male (* $P < 0.05$)

Discussion

Here we assessed the level of expression of thousands of genes, to gain insight into the molecular pathways involved in the coloration of cichlid fish. Our microarray experiment highlighted 46 clones exhibiting differential expression between the two sexes of PS which, despite both having dark vertical stripes, differ greatly in coloration: females have yellow and males bright blue body colorations (Fig. 1a, b). Using qRT-PCR, we amplified 44 of the 46 clones identified in the microarray as well as *csf1ra* and *csf1rb*. Of these, five are good candidates to study the molecular basis of sexual dimorphism in the skin of cichlids, as they have been confirmed to be differentially expressed between males and females (see Fig. 2).

Coatomer Protein Complex, Subunit Zeta-1 (Copz-1)

The resulting candidate gene list is composed of *Collagen 1 alpha*, *Coatomer protein complex*, *subunit zeta-1* (*Copz-1*), one clone that matches to ubiquitin-conjugating enzyme in the 3′-UTR and two clones which do not have any annotated homologues. *Copz-1* is currently the strongest candidate color gene derived from this study. Copz is a subunit of a protein complex that is a precursor of non-clathrin coated Golgi transport vesicles (Waters et al. 1991; Cosson et al. 1996; Moelleken et al. 2007). Previous data have established a connection between pigmentation and intracellular transport defects. Two human diseases, Chediak–Higashi syndrome and Hermansky–Pudlak syndrome, are characterized by albinism as well intracellular traffic disorders. In both diseases, cells exhibit abnormalities in endosomal/lysosomal trafficking in various cytoplasmic organelles including melanosomes (Scriver 1995).

Copz is known to have a conserved role in pigmentation. In *Drosophila*, the altered expression of this gene has been shown to influence pigment granules of the eye (Scriver 1995). In addition, in zebrafish, *Copz* mutants show a reduction in the retinal pigmented epithelium (Gross et al. 2005). In addition, the mutation of three other vesicle trafficking genes in zebrafish is known to result in pronounced hypopigmentation as well as retinal defects (Maldonado et al. 2006). As it is thought that pigment granules are synthesized through transport between the

endosome and the Golgi, Copz might be involved in the yellow coloration of female PS. The production of xanthophores requires the formation of a large number of vesicles for the synthesis and transport of pigments. Mutation of *Copz* in zebrafish does not result in measurably altered skin pigmentation. This implies that the putative role for Copz-1 in cichlid skin pigmentation arose in the lineage leading to the cichlid fishes, after its split from the lineage leading to the zebrafish.

Collagen 1 alpha

Collagen 1 alpha also represents a strong candidate for the sexual dimorphism in the skin of PS, albeit it is unlikely to be related to skin coloration. *Collagen 1 alpha* encodes a structural component of teleost skin and is known to be expressed in the skin of zebrafish throughout development (Le Guellec et al. 2004). Sexual dimorphism in skin thickness is observed in several teleost species (Smith 1978; Burton 1979; Pottinger and Pickering 1985), with the males displaying thicker epidermal and dermal layers due to elevated androgen levels. It is likely that the increased level of *Collagen 1 alpha* expression in the skin of male PS corresponds to a structural difference in the thickness of the epithelium.

We have successfully used microarray to identify promising candidates for sexually dimorphic coloration in cichlid fishes, however, we have met with some technical limitations that are likely to explain our low level of concurrence between the microarray and qRT-PCR data. For example, the use of a single array as a reference may have reduced the robustness of our study. Additionally, the inclusion of biological replication in our qRT-PCR analysis has highlighted that several of the genes in our list display variable levels of expression between individuals. In some cases, additional replicates might have increased the statistical power enough to identify significant differences; however, in other cases the variability is extremely high. This may be due to genuine biological differences, however, they are also likely to be confounded by minor differences in skin sampling regime, in which more or less of the underlying muscle may have been removed. Specifically, five clones (two parvalbumin orthologues, myosin light chain 2, fast/white muscle troponin T and 19C D03, which did not have a characterized orthologue) showed considerable differences in expression that related to individual sample, rather than sex (Supplementary Fig. S2). Their marked variability prevented a statistically significant result from being obtained and precludes them from being considered informative "color genes." Overall, these genes are over-expressed in female versus male samples, explaining their presence in the gene list derived from the microarray, which was based on pooled samples.

Interestingly, we did not detect differential expression of *csf1ra* and *csf1rb*, which were initially included as positive controls. Strong expression of *csf1ra* has previously been demonstrated in newly formed egg dummies on the anal fins of cichlid fishes (Salzburger et al. 2007). Therefore, our results support the involvement of this gene in the specification of new xanthophores, but not their maintenance.

Our experiment has also revealed some limitations of expression profiling in skin samples. For example, we found that *β-actin* and *gapdh* gave inconsistent results when they were used for normalization. This may relate to either sex-biased expression of one of these genes, or minor differences in skin sampling regime, where one may inadvertently include a few muscle fibers along with the skin samples, and skew the expression of genes that are normally up-regulated in muscle cells (see Supplementary Fig. S2). It should be noted that in general it is challenging to obtain high-quality RNA from skin samples, due to the high proportion of inorganic material in the scales and due to difficulties in sampling all layers of skin while avoiding the underlying muscles. The difficulties we have encountered could have been minimized by taking samples from the caudal fin (which is also brightly colored), rather than from the flank. Indeed, this sampling regime has been employed successfully to examine the role of *Pax7* in color patterning for various Lake Malawi cichlids (Roberts et al. 2009). Nevertheless, we have successfully employed a technique that removes the need for normalization, which appears to be a good choice for use in studies of non-model organisms that do not have confirmed control genes (Cummings et al. 2008).

Conclusions

We used complementary microarray techniques and qRT-PCR experiments to discover novel candidate genes that underlie coloration in cichlids. Our study has identified several novel color gene candidates, the most promising of which is involved in endosome-to-Golgi trafficking. Future studies that investigate the function and regulation of these candidate genes will be required to further determine their exact role in pigmentation at the cellular and biochemical level.

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