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Sequence variation in the melanocortin-1 receptor gene (*Mc1r*) does not explain variation in the degree of melanism in a widespread amphibian

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Variation in nucleotide sequence of the melanocortin-1 receptor gene (*Mc1r*) is associated with melanism in several mammalian, avian and reptilian species, but no attempts have been made to understand the genetic underpinnings of melanism in amphibians. We isolated the complete coding sequence (945 bps) of *Mc1r* from the common frog (*Rana temporaria*) and compared the predicted amino acid sequence with that of fish, reptiles, birds and mammals. We investigated associations between nucleotide substitutions and the level of dorsal melanism among 28 individuals from two populations with pronounced differences in melanism. According to our results, the transmembrane regions of *Mc1r* are conserved across vertebrates. In the population comparison, we only found five nucleotide sites with synonymous substitutions; none is being associated with the level of melanism. Our results suggest that either other genes or regulatory regions outside the coding sequence of *Mc1r* are responsible for expression of melanism in *R. temporaria*.

Introduction

Understanding the functional genetic underpinnings of ecologically relevant, fitness-related traits has become one of the main goals in current evolutionary genetic research. In species where detailed genomic knowledge is not available, and/or large-scale breeding experiments are not feasible, candidate-gene analysis provide means to detect genes underlying the variation in phenotypic traits of interest (e.g. Palopoli & Patel 1996). Successful applications of candidate-gene analyses are available among studies focusing on colour variation in animals, particularly with respect to the association between melanism and sequence variation in the melanocortin-1 receptor gene (*Mc1r*) in both domesticated (e.g. Takeuchi *et al*. 1996, Våge *et al*. 1997, Kijas *et al*. 1998) and wild animal populations (mammals: Ritland *et al*. 2001, Eizirik *et al*. 2003, Nachman *et al*. 2003, Hoekstra *et al*. 2006; birds: Theron *et al*. 2001, Mundy *et al*. 2004; reptiles:

Rosenblum *et al*. 2004). Hoekstra *et al*.'s (2006) study showed that even a single amino acid substitution in the melanocortin-1 receptor can lead to adaptive colour variation in the wild.

Previous research on *Mc1r* and colour variability in wild populations had almost exclusively dealt with mammals and birds, the only exception being Rosenblum *et al*.'s (2004) study on reptiles. Besides crypsis or different signalling functions, melanism in ectotherms has a profound effect on their thermal balance with the environment: melanistic individuals heat (or cool) faster than their lighter conspecifics (insects: de Jong *et al*. 1996, Forsman 1997; amphibians: Vences *et al*. 2002; reptiles: Gibson & Falls 1979, Forsman 1995). Because body temperature experienced by an ectotherm during its life strongly affects its fitness via affecting its physiological performance, behaviour and life history (e.g. Van Damme *et al*. 1991, Sinervo & Adolph 1994, Angilletta *et al*. 2002), the functional genetic basis of melanism in different ectothermic taxa surely deserves investigation.

Amphibians represent an interesting group for studying the genetic basis of colour differences due to the enormous colour variation found both within and between species (e.g. Richards & Nace 1983, Hoffman & Blouin 2000). The common frog (*Rana temporaria*) is an especially good candidate for such a study, because it has an extremely wide distribution and inhabits many different habitats in several biomes including both extremely high altitudes and latitudes (e.g. Gasc *et al*. 1997) where it expresses high level of melanism (Vences *et al*. 2002, G. Herczeg unpubl. data). Further, the level of melanism is thought to affect the thermal biology (and may affect UV-tolerance) of this species, which is known as an active behavioural thermoregulator (Vences *et al*. 2002).

The aims of the present study were to provide characterisation of sequence variation of *Mc1r* in an amphibian species, and to test for a possible association between *Mc1r* sequence variation and the level of melanism in *R. temporaria*. For this purpose, we compared *Mc1r* sequence variation and dorsal melanism within and between two *R. temporaria* populations differing in mean and range of dorsal melanism.

Material and methods

Study animals and scoring of melanism

The common frog is a large (approx. 50–80 mm), widespread anuran native to the Palaearctic region. It is one of the anurans with the widest distribution area and can be found in variety of habitats and climates (e.g. Gasc *et al*. 1997). It is an explosive breeder in which mating and egg deposition occurs in aggregated leks right after hibernation (e.g. Wells 1977, Elmberg 1990). The species show adaptive differentiation with respect to many traits along a latitudinal cline across Fennoscandia. For instance, northern frogs are characterized by higher larval developmental rates, relatively smaller testicles and higher prebreeding energy reserves than the southern frogs (Laugen *et al*. 2003, Hettyey *et al*. 2005, Jönsson *et al*. 2009).

Individuals from two Swedish populations (Tvedöra: 55°42´N, 13°26´E, 22 m a.s.l.; and Ammarnäs: 65°54´N, 16°18´E, 410 m a.s.l.) were chosen from a large collection (frozen material) containing samples covering a 1600-km-long Fennoscandian latitudinal gradient (*see* e.g. Hettyey *et al*. 2005, Laugen *et al*. 2005, Jönsson *et al*. 2009). The two populations were chosen in order to maximise both within and between population variation in dorsal melanism (G. Herczeg unpubl. data; *see* Fig. 1). Twenty randomly chosen adult individuals from both Ammarnäs and Tvedöra were used for scoring melanism.

Adult common frogs were collected in 1998 and 1999 at their breeding sites, right after their emergence from hibernation. After capture, animals were kept in water at 6 °C under laboratory conditions and within four days, they were killed by an overdose of MS222 (tricaine methanesulfonate). After this, they were kept in a freezer (–20 °C) until photographed (*see* below) in October 2003.

The frozen frogs were thawed, and digital photographs were taken from their dorsal surfaces. Photographs were taken with a Nikon Coolpix 4500 digital camera (Nikon Corp., Tokyo, Japan) under identical circumstances. The frogs' dorsal surface was gently pressed against a transparent plexiglass panel in order to obtain a

nearly horizontal area for estimating the level of melanism. A ruler was placed in each photograph for scaling. We note that in *R. temporaria* (similarly to many ranids) the visible colour pattern has two components (Fig. 1): (i) the 'base' colour that the individuals can alter relatively quickly and thus it is variable within the same individual, and (ii) the colour pattern caused by the irreversible melanistic spots that is permanent and independent of treatment or individual state (author's pers. obs). We focussed on the latter.

Digital photographs were processed by using the Image-Pro Plus 4.5 software (Media Cybernetics Inc., Bethesda, USA). After defining the colour of the spots of interest, and setting the scale with the aid of the ruler in the photograph, the software automatically calculated from the images the area of each spot and the remaining area. The level of melanism was estimated as the area covered by black spots (only spots > 1 mm^2 were considered in order to exclude noise) divided by the whole measured area (total dorsal surface excluding the limbs). One individual from Tvedöra was excluded due low picture quality.

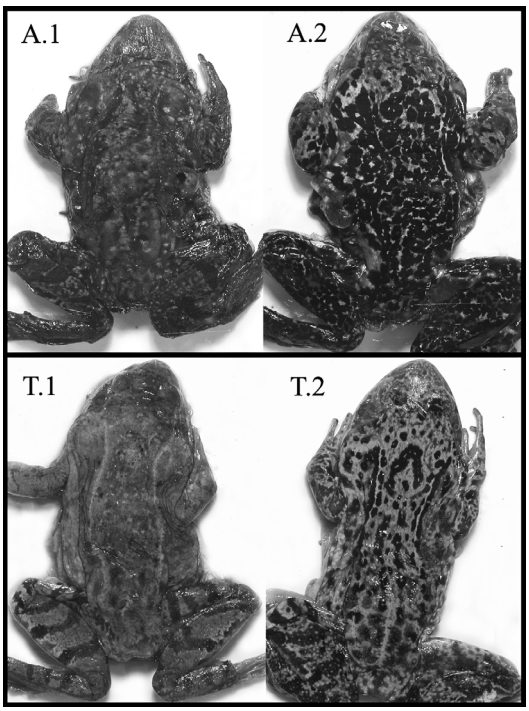

Fig. 1. Representative dorsal colour patterns found in *Rana temporaria*. '1' denotes the lightest and '2' the darkest individual within our population samples. 'A' = Ammarnäs, 'T' = Tvedöra. Note that we did not analyse the (rapidly) changeable base colour, only the relative area covered by the black spots.

Amplification and sequencing of *Mc1r*

To determine a whole *Mc1r* gene sequence of *Rana temporaria*, first, a 645 bps length fragment of the *Mc1r* gene was partially amplified from genomic DNA of a male common frog originating from Härnösand (62°37´N, 17°59´E), Sweden, by PCR using primers *Mc1r*-F1 (5´-GTACTWCTTCATCTGCTGCC) designed on the basis of conserved regions in fish, reptiles and birds (species and Genbank Accession Number [hereafter GAN]: *Takifugu rubripes* AY227791; *Sceloporus undulatus* AY586150; *Uta stansburiana* AY586160; *Anniella pulchra* AY586033; *Aspidoscelis inornata* AY586065; *Holbrookia maculata* AY586106; *Gallus gallus* D78272) and -evoR2 (5´-AAGGCRTAGATGAGGGGGTC; from Rosenblum *et al*. 2004). The 5´- and 3´- parts of the partial sequence were isolated from the *R. temporaria* genome by using a gene-walking PCR technique (DNA Walking SpeedUp premix Kit, Seegene Inc., Seoul, Korea). Using universal anchor included with amplification/sequencing primer site and the nested primers based on first 645 bps fragment, the 5´ and 3´ parts of the *Mc1r* gene were isolated. For gene-walking, nested primers *Mc1r*-fF5 (5´-ATTACTCGGCATCTTCTTCC), -fF6 (5´-CTTCTTCCTGTGCTGGGGGC) and -fF7 (5´-ACCCCTTCTGCCTCCAGTAC) were designed for up-stream walking, and primers *Mc1r*-fR8 (5´-ATGGCTCCCCTTAGTTTGGC), -fR9 (5´-GGAGATGCTCTGCGAATGGC) and -fR10 (5´-GGCAATGGCTCCGAGAAAGG) for down-stream walking. The conditions for each of the steps followed a protocol of the DNA Walking SpeedUp premix Kit.

After obtaining the down- and up-stream sequences of *Mc1r* (GAN: EF165013), common frog specific primers spanning the entire *Mc1r* gene were designed. The 1318 bp fragment containing the whole *Mc1r* ORF (945 bp) was amplified by the frog specific primers *Mc1r*-fF12

(5´-AAGATAATCTGGAGAAATCTCTG) and -fR11 (5´-GCTAAAATCAGGTTGGAATGTC), and sequenced by using primers *Mc1r*-fF12, F-1, -fF5, -fR10 and -fR14 (5´-GCTTCTATTC-CCCCTAACC). As melanism differed considerably between the populations (Ammarnäs frogs having much higher mean and wider range of melanism, *see* Results) we decided to sequence the scored 20 individuals from Ammarnäs and 10 representative individuals (covering the observed variation in melanism) from Tvedöra. DNA was extracted from muscle tissue stored at –20 °C or toe tissue fixed in 70% ethanol using a salt extraction method (Aljanabi & Martinez 1997). One individual from each population had to be excluded due to low DNA quality. Gene amplification was performed in a reaction volume of 20 μl under the following conditions: 20 ng DNA, 0.5 μM primer for each, 0.25 unit Taq polymerase (Bioline Inc., Randolp, MA, USA), 1 × PCR buffer (Bioline Inc.), 1.5 mM $MgCl_2$ and 0.25 mM dNTPs, and 35 cycles of (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 100 s) and an additional extension step at 72 °C for 10 min. Amplified fragments were sequenced, and runs were performed on Megabase 1000 capillary sequencer (Amersham Biosciences, Piscataway, USA). Based on a comparison between the sequenced *R. temporaria Mc1r* and the known *Mc1r* genes of other vertebrates, we selected the coding sequences (945 bps) and used this sequence in the following analyses. Sequences were manually edited and aligned using Sequencher 4.1 software (Gene Codes, Ann Arbor, USA). In the cases where both sequence strands showed double peaks at the same position, we considered that the site is heterozygous (e.g. MacDougall-Shackleton *et al.* 2003, Cheviron *et al.* 2006).

The predicted amino acid sequence of frog *Mc1r* was aligned with other vertebrate *Mc1r* sequences obtained from databases by the program ClustalW (Thompson *et al.* 1997) version 1.83. The MEGA ver. 4 program (Tamura *et al.* 2007) was used to construct the phylogenetic tree of *Mc1r* within vertebrates by the Neighbour Joining, Maximum Parsimony and Minimum Evolution methods, and the PhyML software (Guindon & Gascuel 2003) to obtain a maximum likelihood tree.

Statistical analyses

Variances of melanism in the two populations were compared with Levene's test. As the variances differed (Levene's test: $F_{1,37} = 17.66$, $p < 0.001$), we applied arcsine transformation to homogenise variances which was successful (Levene's test: $F_{1,37} = 1.33$, $p = 0.26$). We compared the populations with one-way ANOVA. For testing the hypothesized association between *Mc1r* variants, we used two-way ANOVA with population and nucleotide type (separately for every variable position) as fixed factors and level of melanism as dependent variable. In order to control for age effects on melanism, we also run two-way ANCOVA with population and nucleotide type (separately for every variable position) as fixed factors, snout-vent length (as an age proxy; hereafter SVL) as a covariate and level of melanism as dependent variable. Altogether, 39 individuals ($N_{\text{Ammarnäs}} = 20$, $N_{\text{Tvedöra}} = 19$) were used to quantify dorsal melanism, and 28 in the molecular genetic analysis ($N_{\text{Ammarnäs}} = 19$, $N_{\text{Tvedöra}} = 9$).

Results

The mean degree of melanism was significantly higher in Ammarnäs than in Tvedöra population ($F_{1,37} = 61.83$, $p < 0.001$; mean ± SD: Ammarnäs = 0.596 ± 0.213; Tvedöra = 0.131 ± 0.149) — a difference that is obvious with plain visual inspection (Fig. 1).

Nineteen out of 20 individuals from Ammarnäs and 9 out of 10 individuals from Tvedöra were successfully sequenced for the 945 bps coding region of *Mc1r* (GAN: EU307942-EU307960 for Ammarnäs and EU307961-EU307969 for Tvedöra). The predicted amino acid sequence (from EU307942) showed 85.16% similarity with chicken *Mc1r* (GAN: Q802F9). Multiple alignment of *Mc1r* predicted amino acid sequences for the common frog and four other vertebrate taxa (fish, reptiles, birds and mammals) from a database (Fig. 2a) showed unique insertion of seven amino acids sequence (210 to 217 a.a.). The transmembrane regions were conserved within the five species analyzed. A phylogenetic analysis of predicted amino acid

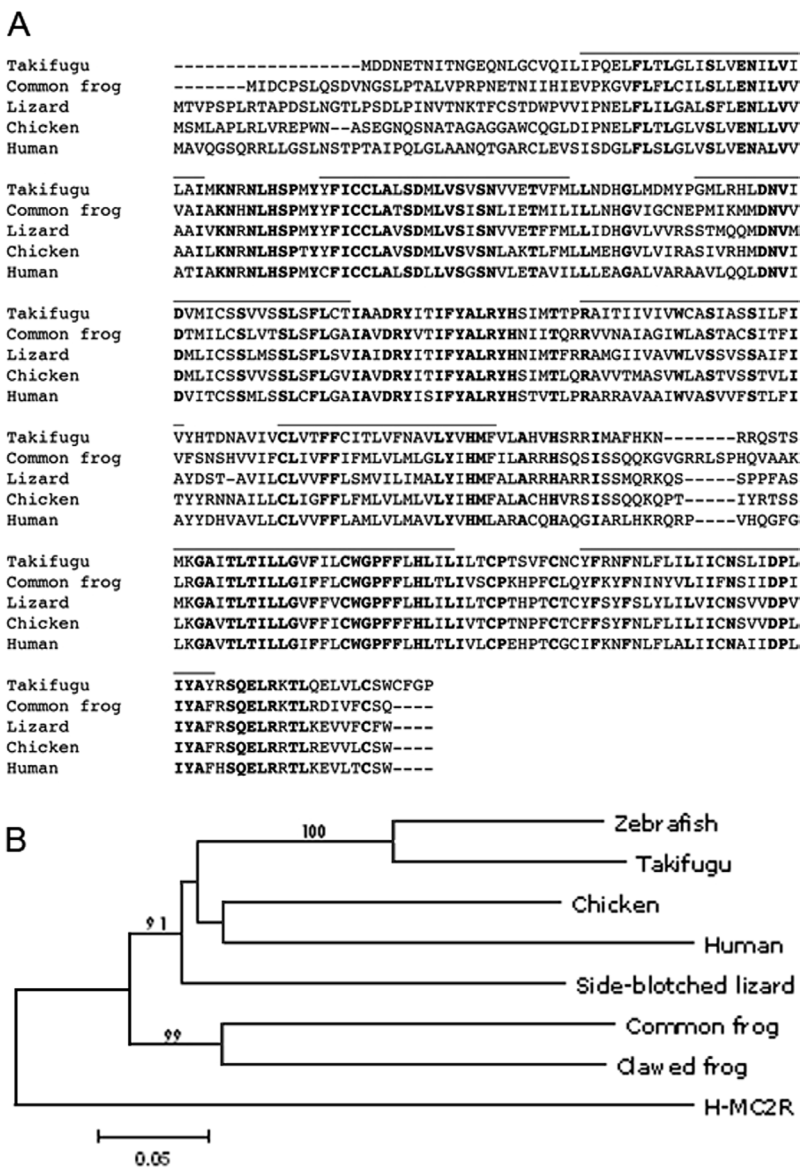

Fig. 2. Alignment of predicted amino acid sequences and phylogenetic tree of vertebrates *Mc1r* genes. The sequences were obtained from database: takifugu (*Takifugu rubripes*, Accession#:AY227791), zebrafish (*Danio rerio*, NM_180970), clawed frog (*Xenopus tropicalis*, scaffold_303:1339944-1340852 from Xentr4), common frog (*Rana temporaria*, from this study), side-blotched lizard (*Uta stansburiana*, AY586160), chicken (*Gallus gallus*, D78272), human (*Homo sapiens*, NM_002386) and human-*Mc2r* (*H. sapiens*, NM_000529). (**A**) A multiple alignment. Solid bars on the sequences indicate seven putative transmembrane regions in *Mc1r* (Klovins *et al.* 2004). Bold letters show conserved amino acids in the sequences between species. (**B**) A Neighbour-Joining tree for amino acid sequences from seven vertebrates. The bootstrap values over 70 are shown on each branch. The bar infers the genetic distance calculated by the Poisson correction method. The human *Mc2r* (H-*Mc2r*) sequence was used as an outgroup.

sequence of seven vertebrate species including fish, amphibian, reptilian, bird and mammalian taxa indicated a specific clade for amphibian *Mc1r* using all four methods (although only Neighbour Joining tree is shown here, the topologies are all same; Fig. 2b).

Five substitution sites were found in total (Table 1), among which two distinct substitu-

tions at nucleotide sites 555 and 594 were found only in one individual from Tvedöra. All substitutions were synonymous; hence, the same protein product is expected from all alleles. However, owing to potential recombination effects with possibly informative regulatory regions closely linked with *Mc1r*, two-way ANOVA (for the variable nucleotide sites 129, 159 and 897) were run to explore possible associations between sequence variation and level of melanism. Melanism differed between populations (all $p < 0.001$), but the nucleotide types (all $p > 0.56$) or the population × nucleotide type interactions (all $p > 0.21$) were not associated with melanism in any of the substitution sites (*see* also Table 1). The results remained qualitatively the same when we controlled for body size (population: all $p > 0.16$; nucleotide type: all $p > 0.32$; population × nucleotide type: all $p > 0.14$; SVL: all $p < 0.01$). Hence, the degree of melanism is not associated with *Mc1r* coding sequence variation in *R. temporaria*.

Discussion

In birds and mammals, increased *Mc1r* activity results in darker colour (by increased eumelanin production), while decreased *Mc1r* activity causes lighter colour (by increased phaeomelanin production) (for review *see* García-Borrón *et al.* 2005). In reptiles, *Mc1r* activity might simply affect the amount of eumelanin produced and stored in the melanophores that are respon-

Table 1. *Mc1r* sequence and colour polymorphism data for 28 adult common frog (*Rana temporaria*) individuals from two populations. All nucleotide substitutions are synonymous. Level of melanism is provided as the area covered by melanistic spots divided by the whole area measured on the dorsal surface; the arcsine transformed values (used in the analyses) are showed in parentheses.

Population	Individual	Level of melanism	Nucleotide site				
			129	159	555	594	897
Tvedöra	1	0.000 (0.000)	C	C	G	C	T
	2	0.005 (0.074)	C/T	C/T	–	–	–
	3	0.009 (0.094)	–	–	–	–	–
	4	0.027 (0.165)	–	–	–	–	–
	5	0.029 (0.172)	C/T	C/T	–	–	C
	6	0.050 (0.226)	C/T	C/T	C/G	C/T	–
	7	0.053 (0.231)	C/T	C/T	–	–	C/T
	8	0.122 (0.367)	–	–	–	–	–
	9	0.199 (0.463)	–	–	–	–	–
Ammarnäs	1	0.003 (0.057)	–	T	–	–	C/T
	2	0.113 (0.342)	T	T	–	–	C/T
	3	0.121 (0.355)	C/T	C/T	–	–	–
	4	0.153 (0.401)	–	C/T	–	–	C/T
	5	0.171 (0.426)	C/T	C/T	–	–	C/T
	6	0.210 (0.476)	T	T	–	–	–
	7	0.220 (0.488)	–	–	–	–	–
	8	0.294 (0.573)	C/T	C/T	–	–	–
	9	0.304 (0.584)	–	–	–	–	–
	10	0.351 (0.634)	C/T	C/T	–	–	C/T
	11	0.375 (0.659)	–	–	–	–	–
	12	0.378 (0.662)	–	–	–	–	–
	13	0.386 (0.671)	C/T	C/T	–	–	–
	14	0.422 (0.707)	T	T	–	–	–
	15	0.429 (0.714)	C/T	C/T	–	–	C/T
	16	0.480 (0.766)	C/T	C/T	–	–	C/T
	17	0.481 (0.766)	C/T	C/T	–	–	C/T
	18	0.640 (0.927)	T	T	–	–	C
	19	0.646 (0.933)	C/T	C/T	–	–	–

sible to the general darkness of the body surface (Rosenblum *et al*. 2004 and references therein). Given the similar mechanism of colour expression of anurans (Hoffman & Blouin 2000), the general role of *Mc1r* could be expected to be similar to that of reptiles. We found that the transmembrane regions of the *Mc1r* protein were conserved across all studied vertebrate taxa. However, our phylogenetic analysis revealed a specific amphibian clade within vertebrates, forming a group separated from fish, reptiles, birds and mammals together. At the intraspecific level, we found no evidence for a link between dorsal colour polymorphism (ranging from 0% to 65% levels of dorsal melanism) and sequence variation at *Mc1r* in *R. temporaria*. Within the 945 bps coding region, we detected five nucleotide sites where substitutions had happened, all being synonymous.

Despite the large number of studies supporting the association of *Mc1r* sequence polymorphism and pigmentation (reviewed in Majerus & Mundy 2003, Mundy 2005), the situation is not always simple; the level of melanism is not necessarily associated with *Mc1r* sequence variation (mammals: Mundy & Kelly 2003, Hosoda *et al*. 2005, Wlasiuk & Nachman 2007; birds: MacDougall-Shackleton *et al*. 2003, Cheviron *et al*. 2006; reptiles: Rosenblum *et al*. 2004). Pigmentation can be determined by other genes with large effects, the agouti signalling protein gene (*agouti*) being one well-known example (e.g. Eizirik *et al*. 2003) or genes with large effects can interact (*Mc1r***agouti*: Steiner *et al*. 2007). Even populations within a single species can differ in the genes causing melanism under presumably similar selective forces (Hoekstra & Nachman 2003). Furthermore, the number of genes known to affect colouration is high; in mice far over 100 genes with effects on colouration have been recognized (Bennett & Lamoreux 2003). Hence, for drawing firm conclusions about the colour altering potential of *Mc1r* mutations in amphibians, several more species with polymorphic colour patterns should be tested.

The dorsal colour pattern is changing during ontogeny in *R. temporaria* with larger (~ older) individuals having higher levels of melanism than smaller (~ younger) ones (Riobo *et al*. 2000; our study). This suggests — similarly to Cheviron *et al*.'s (2006) case with the blue-crowned manakin — that a regulatory gene (or genes) might be involved with expression of melanism. Furthermore, colour polymorphism is not represented by discrete colour phenotypes in *R. temporaria*, rather a continuous shift is observable (Vences *et al*. 2002, author's pers. obs.), suggesting that dorsal colour is a polygenic trait. However, for continuous traits, polymorphisms in the regulatory regions of the gene responsible for the given trait are also conceivable as sources of variation; because such changes would alter the quantity of the gene's product rather than changing its function (for colour *see* e.g. Prud'homme *et al*. 2006). Finally, a merely ontogenetic effect, suntanning, which occurs also in lower vertebrates (Lowe & Goodman-Lowe 1996) cannot be excluded in our case either, even though dark phenotype of ranid frogs seems to be genetically based (Richards & Nace 1983).

In summary, we found that variation in *Mc1r* coding sequence did not predict the dorsal colour pattern in *R. temporaria*, a widespread anuran occurring in high altitudes and latitudes where it has highly melanistic dorsal colour. Considering the high ecological relevance of melanism in ectotherms due to its possible role in thermoregulation, we suggest that further attempts to identify the genes involved in melanism are needed. With respect to amphibians, species with ontogenetically stable, fixed colour morphs (i.e. light-dark) would be preferable models, and/or analysing other candidate genes, including ones with regulatory functions, would be beneficial.

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