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

Gábor Herczeg^{1,*}, Chikako Matsuba^{1,2} & Juha Merilä¹

²⁾ current address: Department of Zoology, University of Florida, 223 Bartram Hall, P.O. Box 118525, University of Florida, Gainesville, FL32611-8525, USA

Received 10 Nov. 2008, revised version received 30 Sep. 2009, accepted 18 Sep. 2009

Herczeg, G., Matsuba, C. & Merilä, J. 2010: Sequence variation in the melanocortin-1 receptor gene (*Mc1r*) does not explain variation in the degree of melanism in a widespread amphibian. — *Ann. Zool. Fennici* 47: 37–45.

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.* 2001, Hoekstra *et al.* 2006; birds: Theron *et al.* 2001, Mundy *et al.* 2004; reptiles:

¹⁾ Ecological Genetics Research Unit, Department of Biological and Environmental Sciences, P.O. Box 65, FI-00014 University of Helsinki, Finland (*corresponding author's e-mail: gabor. herczeg@helsinki.fi)

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 mm² 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.

Amplification and sequencing of Mc1r

To determine a whole *Mc1r* gene sequence of Rana temporaria, first, a 645 bps length fragment of the Mclr 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'-AAGGCRTAGAT-GAGGGGGGTC; 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



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.

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'-CTTCTTCCTGTGCTGGGGGGC) 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 McIr (GAN: EF165013), common frog specific primers spanning the entire McIr gene were designed. The 1318 bp fragment containing the whole McIr ORF (945 bp) was amplified by the frog specific primers McIr-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 μ l under the following conditions: 20 ng DNA, $0.5 \mu M$ primer for each, 0.25 unit Taq polymerase (Bioline Inc., Randolp, MA, USA), $1 \times PCR$ buffer (Bioline Inc.), 1.5 mM MgCl₂ 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 Mclr and the known Mclr 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: $F_{137} = 17.66$, 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 ($N_{Ammannäs}$ = $19, N_{\text{Tyedöra}} = 9).$

Results

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

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 Mclr (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 Mclr, 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 \times 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)	С	С	G	С	Т
	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	-	_	С
	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)	-	Т	_	_	C/T
	2	0.113 (0.342)	Т	Т	-	_	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)	Т	Т	-	_	-
	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)	Т	Т	_	_	-
	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)	Т	Т	-	-	С
	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 Mclr 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 Mclr 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 Chevi-

ron 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.

Acknowledgements

Our study was supported by Academy of Finland (HG, JM) and Otto A. Malmi foundation (CM). Thanks to Ingemar Jönsson and Maarit Haataja for their help in obtaining the samples, and Erica Leder for her constructive comments on the manuscript.

References

- Aljanabi, S. M. & Martinez, I. 1997: Universal and rapid saltextraction of high quality genomic DNA for PCR-based techniques. — *Nucleic Acids Research* 25: 4692–4693.
- Angilletta, M. J. Jr., Hill, T. & Robson, M. A. 2002: Is

physiological performance optimized by thermoregulatory behavior?: a case study of the eastern fence lizard, *Sceloporus undulatus. — Journal of Thermal Biology* 27: 199–204.

- Bennett, D. C. & Lamoreux, M. L. 2003: The color loci of mice – a genetic century. – *Pigment Cell Research* 16: 333–344.
- Cheviron, Z. A., Hackett, S. J. & Brumfield, R. T. 2006: Sequence variation in the coding region of the melanocortin-1 receptor gene (*MC1R*) is not associated with plumage variation in the blue-crowned manakin (*Lepidopthrix coronata*). — *Proceedings of the Royal Society B* 273: 1613–1618.
- de Jong, P. W., Gussekloo, S. W. S. & Brakefield, P. M. 1996: Differences in thermal balance, body temperature and activity between non-melanic and melanic two-spot ladybird beetles (*Adalia bipunctata*) under controlled conditions. – *Journal of Experimental Biology* 199: 2655–2666.
- Elmberg, J. 1990: Long-term survival, length of breeding season, and operational sex ratio in a boreal population of common frogs, *Rana temporaria* L. – *Canadian Journal of Zoology* 68: 121–127.
- Eizirik, E., Yuhki, N., Johnson, W. E., Menotti-Raymond, M., Hannah, S. S. & O'Brien, S. J. 2003: Molecular genetics and evolution of melanism in the cat family. – *Current Biology* 13: 448–453.
- Forsman, A. 1995: Heating rates and body temperature variation in melanistic and zigzag *Vipera berus*: does colour make a difference? — *Annales Zoologici Fennici* 32: 365–374.
- Forsman, A. 1997: Thermal capacity of different colour morphs in the pygmy grasshopper *Tetrix subulata*. — *Annales Zoologici Fennici* 34: 145–149.
- García-Borrón, J. C., Sánchez-Laorden, B. L. & Jiménez-Cervantes, C. 2005: Melanocortin-1 structure and functional regulation. — *Pigment Cell Research* 18: 393–410.
- Gasc, J.-P., Cabela, A., Crnobrnja-Isailovic, J., Dolmen, D., Grossenbacher, K., Haffner, P., Lescure, J., Martens, H., Martínez Rica, J. P., Maurin, H., Oliveira, M. E., Sofianidou, T. S., Veith, M. & Zuiderwijk, A. (eds.) 1997: *Atlas of amphibians and reptiles in Europe*. — Societas Europea Herpetologica & Museum National d'Histoire Naturelle (IEGB/SPN), Paris.
- Gibson, A. R. & Falls, J. B. 1979: Thermal biology of the common garter snake *Thamnophis sirtalis* (L.). II. The effects of melanism. — *Oecologia* 43: 99–109.
- Guindon, S. & Gascuel, O. 2003: PhyML A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. – *Syst. Biol.* 52: 696–704.
- Hettyey, A., Laurila, A., Herczeg, G., Jönsson, K. I., Kovács, T. & Merilä, J. 2005: Does testis weight decline towards the Subarctic? A case study on the common frog, *Rana temporaria*. – *Naturwissenschaften* 92: 188–192.
- Hoekstra, H. E. & Nachman, M. W. 2003: Different genes underlie adaptive melanism in different populations of pocket mice. — *Molecular Ecology* 12: 1185–1194.
- Hoekstra, H. E., Hirschmann, R. J., Bundey, R. A., Insel, P. A. & Crossland, J. P. 2006: A single amino acid mutation

contributes to adaptive beach mouse color pattern. — *Science* 313: 101–104.

- Hoffman, E. A. & Blouin, M. S. 2000: A review of colour and pattern polymorphisms in anurans. — *Biological Journal of the Linnean Society* 70: 633–665.
- Hosoda, T., Sato, J. J., Shimada, T., Campbell, K. L. & Suzuki, H. 2005: Independent nonframeshift deletions in the *MC1R* gene are not associated with melanistic coat coloration in three mustelid lineages. — *Journal of Heredity* 96: 607–613.
- Jönsson, K. I., Herczeg, G., O'Hara, R. B., Söderman, F., ter Shure, A., Larsson, P. & Merilä, J. 2009: Sexual patterns of prebreeding energy reserves in the common frog *Rana temporaria* along a latitudinal gradient. — *Ecography* 5: 831–839.
- Kijas, J. M. H., Wales, R., Törnsten, A., Chardon, P., Moller, M. & Andersson, L. 1998: Melanocortin receptor 1 (*MC1R*) mutations and coat color in pigs. – *Genetics* 150: 1177–1185.
- Klovins, J., Haitina, T., Fridmanis, D., Kilianova, Z., Kapa, I., Fredriksson, R., Gallo-Payet, N. & Schiöth, H. B. 2004: The melanocortin system in fugu: determination of POMC/AGRP/MCR gene repertoire and systeny, as well as pharmocology and anatomical distribution of the MCRs. — *Molecular Biology and Evolution* 21: 563–579.
- Laugen, A. T., Laurila, A., Räsänen, K. & Merilä, J. 2003: Latitudinal countergradient variation in the common frog (*Rana temporaria*) developmental rates — evidence for local adaptation. — *Journal of Evolutionary Biology* 16: 996–1005.
- Laugen, A. T., Jönsson, K. I., Laurila, A., Söderman, F. & Merilä, J. 2005: Do common frogs (*Rana temporaria*) follow Bergman's rule? — *Evolutionary Ecology Research* 7: 717–731.
- Lowe, C. & Goodman-Lowe, G. 1996: Suntanning in hammerhead sharks. — *Nature* 383: 677.
- MacDougall-Shackleton, E. A., Blanchard, L. & Gibbs, H. L. 2003: Unmelanized plumage patterns in old world leaf warblers do not correspond to sequence variation at the melanocortin-1 receptor locus (*MC1R*). — *Molecular Biology and Evolution* 20: 1675–1681.
- Majerus, M. E. N. & Mundy, N. I. 2003: Mammalian melanism: natural selection in black and white. — *Trends in Genetics* 19: 585–588.
- Mundy, N. I. 2005: A window on the genetics of evolution: MCIR and plumage colouration in birds. — Proceedings of the Royal Society B 272: 1633–1640.
- Mundy, N. I. & Kelly, J. 2003: Evolution in a pigmentation gene, the melanocortin-1 receptor, in primates. — American Journal of Physical Anthropology 121: 67–80.
- Mundy, N. I., Badcock, N. S., Hart, T., Scribner, K., Janssen, K. & Nadeau, N. J. 2004: Conserved genetic basis of a quantitative plumage trait involved in mate choice. — *Science* 303: 1870–1873.
- Nachman, M. W., Hoekstra, H. E. & D'Agostino, S. L. 2003: The genetic basis of adaptive melanism in pocket mice. — Proceedings of the National Academy of Sciences USA 100: 5268–5272.
- Palopoli, M. F. & Patel, N. H. 1996: Neo-Darwinian develop-

mental evolution: can we bridge the gap between pattern and process? — *Current Opinion in Genetics and Development* 6: 502–508.

- Prud'homme, B., Gompel, N., Rokas, A., Kassner, V. A., Williams, T. M., Yeh, S.-D., True, J. R. & Carrol, S. B. 2006: Repeated morphological evolution through cisregulatory changes in a pleiotropic gene. — *Nature* 440: 1050–1053.
- Richards, C. M. & Nace, G. W. 1983: Dark pigment variants in anurans: classification, new descriptions, color changes and inheritance. – *Copeia* 1983: 979–990.
- Riobó, A., Rey, J., Puente, M., Miramontes, C. & Vences, M. 2000: Ontogenetic increase of black dorsal pattern in *Rana temporaria*. — *British Herpetological Society Bulletin* 70: 1–6.
- Ritland, K., Newton, C. & Marshall, H. D. 2001: Inheritance and population structure of the white-phased "Kermode" bear. – *Current Biology* 11: 1468–1472.
- Rosenblum, E. B., Hoekstra, H. E. & Nachman, M. W. 2004: Adaptive reptile colour variation and the evolution of the *MC1R* gene. – *Evolution* 58: 1794–1808.
- Sinervo, B. & Adolph, S. C. 1994: Growth plasticity and thermal opportunity in *Sceloporus* lizards. – *Ecology* 75: 776–790.
- Steiner, C. C., Weber, J. N. & Hoekstra, H. E. 2007: Adaptive variation in beach mice produced by two interacting pigmentation genes. – *PLoS Biology* 5: 1880–1889.
- Takeuchi, S., Suzuki, J., Yabuuchi, M. & Takahashi, S. 1996: A possible involvement of melanocortin 1-receptor in regulating feather color pigmentation in chicken. — *Biochimica et Biophysica Acta — Gene Structure and Expression* 1308: 164–168.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007: MEGA4:

Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. — *Moecular Biology and Evolution* 24: 1596–1599.

- Theron, E., Hawkins, K., Bermingham, E., Ricklefs, R. E. & Mundy, N. I. 2001: The molecular basis of an avian plumage polymorphism in the wild: A melanocortin-1 receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*. – *Current Biology* 11: 550–557.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. — Nucleic Acids Research 22: 4673–4680.
- Van Damme, R., Bauwens, D. & Verheyen, R. F. 1991: The thermal dependence of feeding behaviour, food consumption and gut-passage time in the lizard *Lacerta vivipara* Jacquin. — *Functional Ecology* 5: 507–517.
- Våge, D. I., Lu, D., Klungland, H., Lien, S., Adalsteinsson, S. & Cone, R. D. 1997: A non-epistatic interaction of agouti and extension in the fox, *Vulpes vulpes. — Nature Genetics* 15: 311–315.
- Vences, M., Galan, P., Vieites, D. R., Puente, M., Oetter, K. & Wanke, S. 2002: Field body temperatures and heating rates in a montane frog population: the importance of black dorsal pattern for thermoregulation. — *Annales Zoologici Fennici* 39: 209–220.
- Wells, K. D. 1977: The social behavior of anuran amphibians. — Animal Behaviour 25: 666–693.
- Wlasiuk, G. & Nachman, M. W. 2007: The genetics of adaptive coat color in gophers: coding variation in *MC1R* is not responsible for dorsal color differences. — *Journal* of *Heredity* 98: 567–574.