JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02359.x

Association of *Mc1r* variants with ecologically relevant phenotypes in the European ocellated lizard, *Lacerta lepida*

V. L. NUNES*, A. MIRALDO†, M. A. BEAUMONT‡§, R. K. BUTLIN¶ & O. S. PAULO*

*Computational Biology and Population Genomics Group, Centro de Biologia Ambiental, DBA/FCUL, Lisboa, Portugal
†Metapopulation Research Group, Department of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland
‡School of Animal and Microbial Sciences, University of Reading, Reading, UK
§Schools of Mathematics and Biological Sciences, University of Bristol, Bristol, UK
¶Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK

Keywords:

adaptation;
candidate genes;
colour variation;
contact zone;
molecular evolution;
natural selection;
population genetics.

Abstract

A comprehensive knowledge on the genetic basis of coloration is crucial to understand how new colour phenotypes arise and how they contribute to the emergence of new species. Variation in *melanocortin-1 receptor* (*Mc1r*), a gene that has been reported as a target for repeated evolution in a wide range of vertebrate taxa, was assessed in European ocellated lizards (*Lacerta lepida*) to search for associations with melanin-based colour phenotypes. *Lacerta lepida* subspecies' distribution is associated with the three major bio-climatic regions in the Iberian Peninsula. A nonconserved and derived substitution (T162I) was associated with the *L. l. nevadensis* phenotype (prevalence of brown scales). Another substitution (S172C) was associated with the presence of black scales in both *L. l. lepida* and *L. l. iberica*, but no mutations were found to be associated with the higher proportion of black in *L. l. iberica*. Extensive genotyping of *Mc1r* along the contact zone between *L. l. nevadensis* and *L. l. lepida* revealed low gene flow (only two hybrids detected). The implications of these findings are discussed in the context of previous knowledge about the evolutionary history of ocellated lizards.

Introduction

Species and subspecies were once described based on morphological traits alone. Nowadays, the use of molecular tools has become widespread in species other than model organisms, allowing the investigation into the genetic basis underlying morphological variation and its evolution. Coloration has been one of the most extensively studied traits at the molecular level (Hoekstra, 2006). Variation in colour within and between species often has important implications for adaptation to the environment such as in concealment, thermoregulation, mimicry and warning signals (Roulin, 2004; Protas & Patel, 2008). Mammals' and birds' coat colour depends on the amount of eumelanin (black/brown) and phaeomelanin (red/yellow) pigments, produced in melanocytes (Hoekstra, 2006). In poikilothermic vertebrates, besides melanophores (pigment cells able to produce eumelanin, but not phaeomelanin as in mammals), two additional pigment cell types contribute to the diversity of colours and patterns visible to the human eye: xanthophores/erythrophores (yellow to red carotenoid or pteridine pigments) and light-reflecting iridophores (iridescent or silvery structural colours) (Grether *et al.*, 2004). The spatial arrangement and architectural combination of each type of pigment cells determine the skin colour of reptiles, fishes and amphibians (Grether *et al.*, 2004).

Genes and pathways underlying nonmelanin-based colour patterns in poikilothermic vertebrates remain poorly understood (Hubbard *et al.*, 2010). The melanin synthesis pathway is an exception, as it is quite conserved among vertebrates, and extensive study in model species has led to more than 100 well-characterized genes with known effects on coloration (Hoekstra, 2006). *Melanocortin-1 receptor* (*Mc1r*), in particular, has been extensively studied to date, and associations between changes in its

Correspondence: Vera L. Nunes, Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa, Edifício C2-2.3.22, Campo Grande, 1749-016 Lisboa, Portugal.
Tel.: +351 217500000; fax: +351 217500028;
e-mail: vlnunes@fc.ul.pt

coding sequence and differences in pigmentation have been documented in more than 20 vertebrate species, mostly mammals and birds (Gompel & Prud'homme, 2009). Because *Mc1r* is a small intronless gene of about 950 base pairs (bp) and functionally conserved among vertebrates, there has been a bias towards its study over other candidate genes underlying coloration (Mundy, 2005; Gompel & Prud'homme, 2009). Nevertheless, growing evidence suggests that *Mc1r* may in fact be a recurrent target for evolutionary changes with effect on pigmentation (Gompel & Prud'homme, 2009), being responsible for convergent phenotypes in closely, but also in distantly, related species like mice and mammoths (Manceau *et al.*, 2010). *Mc1r* function is specialized for melanin synthesis, and the high number of variants associated with body colour indicates that many amino acid substitutions are likely to affect protein function without deleterious consequences (Mundy, 2005; Gompel & Prud'homme, 2009). Recent studies provided evidence for the causal effects of *Mc1r* mutations on melanin production by functional assays in cavefish *Astyanax mexicanus* (Gross *et al.*, 2009) and in lizards *Aspidoscelis inornata* and *Sceloporus undulatus* (Rosenblum *et al.*, 2010), reinforcing the importance of *Mc1r* as a candidate gene for coloration in vertebrate groups other than mammals or birds.

Many lizard species are colour polymorphic, enabling an efficient camouflage within the background against visual predators. The species considered in this study, *Lacerta lepida* (synonym of *Timon lepidus* according to Mayer & Bischoff (1996)), is such an example. *Lacerta lepida* is presently the only ocellated lizard species inhabiting the European continent. It is widespread throughout the Iberian Peninsula, extending its range into some discontinuous areas in southern France and northern Italy. Two closely related ocellated lizard species are found in the north of Africa: *Lacerta pater* and *Lacerta tangitana*. In the Iberian Peninsula, three *L. lepida* subspecies were initially recognized based on morphological divergence alone: *L. lepida lepida*, *L. lepida nevadensis* and *L. lepida iberica* (Mateo & Castroviejo, 1990). Their taxonomy was later supported by molecular, cytogenetic and genetic evidence (Mateo *et al.*, 1996, 1999; Paulo *et al.*, 2008; Miraldo *et al.*, 2011). Each *L. lepida* subspecies presents a distinct colour phenotype and occurs in association with a single bioclimatic region of the species' distribution (Mateo & Castroviejo, 1990). The nominal subspecies, *L. l. lepida*, occurs almost all over the species distribution area, in typical Mediterranean habitats. Its dorsal coloration is composed of an irregular pattern of black scales over a green to yellowish background, where green/yellow scales are enclosed by black scales in more or less well-defined circles (Fig. S1, Supporting information). The nominal subspecies is replaced by *L. l. iberica* in the north-west of the Iberian Peninsula, in a region affected by the Atlantic climate, with mild temperatures and abundant annual rainfall. *Lacerta l. iberica* has a smaller body size and darker dorsal coloration, with predominance of black scales (Mateo & Castroviejo, 1990). The third subspecies, *L. l. nevadensis*, replaces *L. l. lepida* on the south-eastern coast of the Peninsula. This region is characterized by a reduced and irregular amount of annual precipitation (< 300 mm), which leads to an increased aridity with sparse shrub-like vegetation and large portions of exposed soil (Mateo & Castroviejo, 1990; Hodar *et al.*, 1996). Coloration of *L. l. nevadensis* is remarkably distinct, with a reduced or faded pattern of green/yellow scales and a dominance of brown rather than black scales, producing a phenotype that blends in with the dry landscape (Mateo & Castroviejo, 1990; Mateo & López-Jurado, 1994).

Investigations into genes underlying coloration polymorphism in reptiles have so far been limited to *Mc1r* (Rosenblum *et al.*, 2004; Raia *et al.*, 2010). The characterization of the *Mc1r* locus in three White Sands lizard species (*Aspidoscelis inornata*, *Sceloporus undulatus* and *Holbrookia maculata*) highlighted a single derived amino acid replacement in each species associated with blanched phenotypes (Rosenblum *et al.*, 2004), leading to partial loss of function of *Mc1r* in *A. inornata* and *S. undulatus* (Rosenblum *et al.*, 2010). Here, we investigate *Mc1r* variation in ocellated lizards to shed light on the genetic basis of melanin-based colour phenotypes found in the Iberian Peninsula and contribute to disentangling the importance of *Mc1r* in the evolution of ocellated lizard coloration. Special attention has been given to the variation of *Mc1r* in the vicinity of the contact zone between the nominal subspecies and *L. l. nevadensis*, as this pair exhibits the most contrasting colour phenotypes.

Materials and methods

Colour phenotype characterization

The colour phenotype of European ocellated lizards was characterized through the visual inspection of film photographs from 1997 of the dorsal pattern of 82 adult lizards. The animals were captured at six locations in the Iberian Peninsula: Toledo, Béjar, Castro Marim and Peniche (within *L. l. lepida* distribution range), Galicia (*L. l. iberica*) and Almeria (*L. l. nevadensis*; Fig. 1). Each lizard was placed in a box with a glass cover to fully expose the dorsal side of the animal. A piece of millimetric paper was attached to the glass as size reference. Photographs were taken with a Canon A1 camera (Canon Inc., Tokyo, Japan) using 35-mm Kodak Gold film. Tail tissue samples were collected from the individuals mentioned above and from 19 additional lizards from Serra da Estrela (*L. l. lepida*), Gerês (*L. l. iberica*), Azrou (*L. tangitana*) and Tabarka (*L. pater*; Fig. 1), but no photographic record is available from these. All animals were immediately released back into the wild.

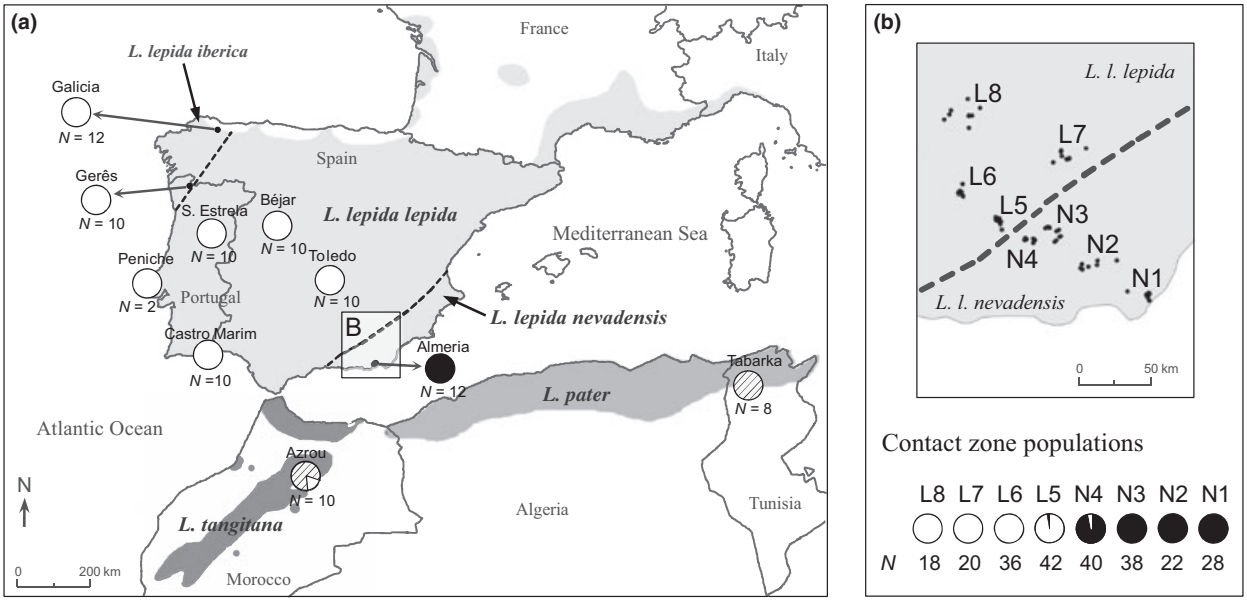

Fig. 1 Map of the western Mediterranean region (a) showing the distribution of ocellated lizards: *Lacerta lepida* (Europe), *L. tangitana* (Morocco) and *L. pater* (Algeria and Tunisia). In the Iberian Peninsula, known distribution limits of *L. lepida* subspecies (*L. l. iberica*, *L. l. lepida* and *L. l. nevadensis*) based on morphology are indicated by dashed lines. A transect of sampled locations across the contact zone between *L. l. nevadensis* (N1–N4) and *L. l. lepida* (L5–L8) is presented in more detail in the zoom box (b). In (a) and (b), pie charts denote the proportion of sampled alleles (*N*) in each location that correspond to each *Melanocortin-1 receptor* haplotype, based on three single-nucleotide polymorphisms, at positions 477 (synonymous), 485 and 514 (nonsynonymous): G-C-T (white), A-T-A (black) and A-C-A (stripped pattern).

To characterize the prevalence of melanin-based colours (black/brown) in lizards' dorsal patterns, all scales present in 1 cm^2 of the mid-dorsal region of each individual were counted and classified according to their colour in one of three possible categories: black, brown or green/yellow scales. The partial melanization index (Mateo, 1988) was calculated as the ratio of the number of black scales over the number of green/yellow scales in 1 cm^2 of the mid-dorsal region. Phenotypic data from both sexes were treated together because no significant sexual dimorphism in dorsal pattern has been described to date (Mateo, 1988; Font *et al.*, 2009). Sample sizes by sex are very small for some populations, but our data reveals no significant differences in counts of scales between males and females (Fisher's exact test, $P > 0.05$), although body size, and as a consequence scale size, is generally higher in males.

Mc1r genotyping and analyses

Whole genome DNA was extracted from tail tissue using Jetquick Tissue DNA kit (Genomed, Löhne, Germany). An additional set of 122 DNA samples was made available from the work of Miraldo (2009), belonging to eight locations (located 20–50 km apart from each other) along a transect perpendicular to the putative contact zone between *L. l. lepida* and *L. l. nevadensis* subspecies in the south-east of the Iberia Peninsula (locations N1–N4 and L5–L8 from Fig. 1). Populations N1 to N4 are fixed for *L. l. nevadensis* haplotypes and populations L5 to L8 are fixed for *L. l. lepida* haplotypes in cytochrome *b* (Miraldo, 2009). DNA samples from other Lacertidae species from the Iberian Peninsula, available from previous studies, were used as outgroups for *Mc1r* analysis: two samples from *L. schreiberi* (Paulo *et al.*, 2001), four from *Iberolacerta monticola* (Moreira *et al.*, 2007) and one from *L. agilis* (Paulo *et al.*, 2008).

A central portion of 450 bp from the *Mc1r* gene was successfully amplified with primers F2 (5′-TACTACTTCATCTGCTGCCTGGC-3′) and R1 (5′-CCCAGSAGGATGGTGAGGGTG-3′) (Rosenblum *et al.*, 2004). PCRs were performed in 25 μL of total volume with 1× PCR buffer (Promega), 1 U *Taq* polymerase (Promega, Madison, WI, USA), 2.0 mM $MgCl_2$, 0.12 mM dNTPs and 0.4 μM of each primer. The cycling conditions used were 94 °C for 3 min, 35 × (94 °C for 30 s, 60 °C for 45 s and 72 °C for 60 s) and 72 °C for 10 min. Purified products (Sureclean; Bioline, London, UK) were sequenced in both directions for a subset of 37 samples, representative of all sampled locations, using standard protocols (BigDye Terminator v.3.1; Applied Biosystems Foster City, CA, USA) on an ABI PRISM 310 (Applied Biosystems). Sequences were edited in Sequencher v.4.0.5 (Gene Codes Co., Ann Arbor, MI, USA) and deposited in GenBank (accession numbers JF732930–JF732966). PHASE 2.1.1 (Stephens *et al.*, 2001; Stephens & Scheet, 2005) was used to infer haplotype phase of six individuals that were heterozygous at multiple sites. We ran the

algorithm five times (1000 iterations with the default values) with different random number seeds, and the same haplotypes were consistently recovered in each run.

Alignments with other *Mc1r* sequences from other vertebrates available in GenBank were performed with CLUSTAL W (Thompson *et al.*, 1994). These included 11 lizard species (*Podarcis bocagei* GU180965, *Aspidoscelis inornata* AY586066, *Mabuya wrightii* GU180949, *Phelsuma astriata* GU180957, *Tarentola mauritanica* HM014691, *Urocotyledon inexpectata* GU180930, *Phrynosoma platyrhinos* AY586113, *Holbrookia maculata* AY586106, *Sceloporus undulatus* AY586127, *Uta stansburiana* AY586159, *Anniella pulchra* AY586034), three snake species (*Thamnophis sirtalis* AY586157, *Morelia boeleni* FJ865133, *Crotalus tigris* EU526278), one bird (*Gallus gallus* AY220305), one fish (*Takifugu rubripes* AY227791) and one mammal (*Bos taurus* AF445641).

Nucleotide diversity (π) and haplotype diversity (H) were determined for each ocellated lizard species and subspecies in DNAsp v5.10 (Librado & Rozas, 2009). Linkage disequilibrium and tests of neutrality were also performed in DNAsp. Neutrality was tested with Tajima's D test (Tajima, 1989) for each ocellated lizard species or subspecies, and the McDonald–Kreitman test (McDonald & Kreitman, 1991) was used to search for selection signatures in three pairs of pooled samples: (i) *L. l. nevadensis* × *L. pater*, (ii) *L. l. nevadensis* × *L. tangitana* and (iii) *L. l. nevadensis* × *L. l. lepida* and *L. l. iberica*. To infer the relationships among haplotypes, a minimum spanning network was constructed with the median-joining method (Bandelt *et al.*, 1999) in NETWORK 4.51 (http://www.fluxus-engineering.com). The input file was converted from fasta to nexus format with CONCATENATOR (Pina-Martins & Paulo, 2008).

Further investigations concerning the geographical distribution of *Mc1r* variants across *L. lepida*'s range, particularly at the contact zone between *L. l. lepi*da and *L. l. nevadensis*, were conducted by genotyping the remaining set of DNA samples (139 lizards) for three single-nucleotide polymorphisms (SNPs) at sites 477, 485 and 514, suspected to be diagnostic for *L. l. nevadensis*. Genotypes were obtained from single-strand sequences of *Mc1r*, using the same protocol as above. Samples suspected to be heterozygous for any of the SNPs were sequenced for the complementary strand to confirm their genotype. Associations between SNP genotypes and colour phenotypes were tested with contingency tables using Fisher's exact test (Fisher, 1935) in R (http://www.R-project.org).

Results

Colour phenotype

Despite individual variation in dorsal colour pattern, it is possible to visually recognize differences in melanin-based colours (black/brown) associated with each of the *Lacerta lepida* subspecies (Fig. S1, Supporting information). The colour phenotype observed in *L. l. nevadensis* individuals from Almeria shows the most conspicuous differences from all others. Unlike populations from *L. l. lepida* and *L. l. iberica*, all *L. l. nevadensis* individuals analysed lack green scales on the head, the hind legs and the tail, having only brown/grey scales on these body parts. Another characteristic trait of the *L. l. nevadensis* phenotype is the tendency for a decrease in the green dorsal pattern. Among the eight lizards analysed from Almeria, green scales in the dorsal pattern clearly reduce in number or disappear completely near the hind leg insertions and the neck in six individuals, leaving the geometrical figures formed by the combination of dark and green scales faded or absent. The same faded pattern was not observed in lizards from the *L. l. lepida* or *L. l. iberica* subspecies. The partial melanization index resulted in contrasting values for each subspecies: 0.56 in *L. l. nevadensis*, 1.36–1.94 in *L. l. lepida* and 3.33 in *L. l. iberica* (Fig. 2). These values reflect the differences in black scale proportions between subspecies. The *L. l. nevadensis* phenotype corresponds to the lowest proportion of black scales (19.6%), as these are replaced by brown scales (43.7%), whereas in the *L. l. iberica* phenotype, the proportion of black scales reaches the highest value (74.6%; Fig. 3).

Mc1r sequence diversity

Sequences of the *Mc1r* gene of 450 bp were obtained from 30 ocellated lizards from Europe and Africa and

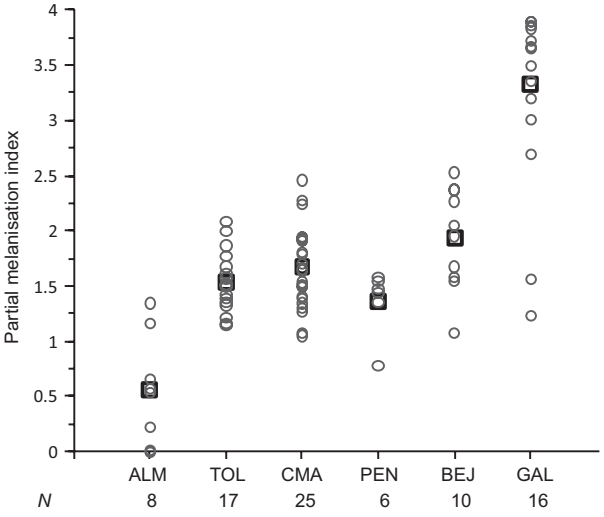

Fig. 2 Partial melanization index (PMI) from six populations of *Lacerta lepida* (N = sample size): Almeria (ALM) from *L. l. nevadensis*, Toledo (TOL), Castro Marim (CMA), Peniche (PEN) and Béjar (BEJ) from *L. l. lepida* and Galicia (GAL) from *L. l. iberica*. Average values of PMI are represented by open squares. The PMI was calculated as the ratio of black scales over light scales (green or yellow) counted in 1 cm^2 of mid-dorsal skin.

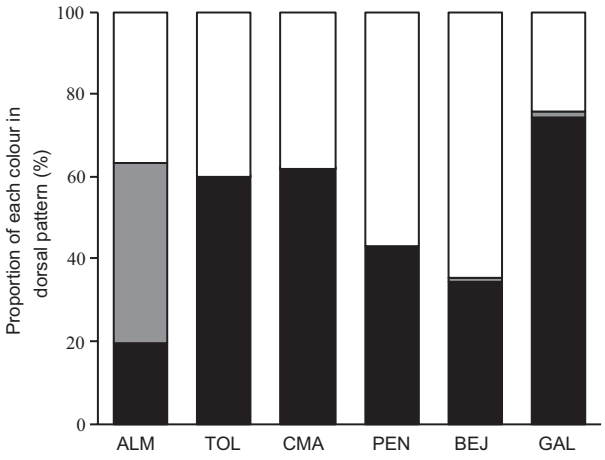

Fig. 3 Average proportion per population of black (in black), brown (in grey) and green/yellow (in white) scales counted in 1 cm^2 of mid-dorsal skin in European ocellated lizards. Each bar represents one population (sample size as in Fig. 2). Almeria (ALM) belongs to *L. l. nevadensis*, Galicia (GAL) is from *L. l. iberica* and the remaining populations are from *L. l. lepida* subspecies.

from another seven lacertid lizards from the Iberian Peninsula. Sequences aligned without gaps from nucleotide 268 to 717 of the 945-bp *Mc1r* gene from little striped whiptail lizard, *Aspidoscelis inornata*, from North America, one of the most closely related species available in GenBank (AY586066) with the full gene sequence. Nucleotide diversity values were similar for the European species *L. lepida* ($\pi = 0.00656$) and the African species *L. pater* ($\pi = 0.00637$) and *L. tangitana* ($\pi = 0.00514$), but when considering each *L. lepida* subspecies alone, *L. l. nevadensis* showed the highest value ($\pi = 0.00333$; Table 1). The highest haplotype diversity was observed in *L. pater* ($H = 0.933$), whereas *L. lepida* showed a lower value ($H = 0.748$), very close to the value observed in *L. l. nevadensis* alone ($H = 0.742$; Table 1).

We observed 13 segregating sites in *Mc1r* from European ocellated lizards, two of them corresponding to nonsynonymous mutations, at positions 485 and 514. The mutation at site 485 was a C-T transition in the second position of codon 162 (T162I) that leads to the replacement of a threonine for an isoleucine residue in the second intracellular protein domain, involving changes in its polarity. A threonine residue at this position was found in all ocellated lizards and other lacertid species investigated, except in *L. l. nevadensis*, where only isoleucine was found (Fig. 4). The second nonsynonymous mutation, at site 514, was located only 29 base pairs away from the mutation T162I and corresponds to an A-T transversion in the first position of codon 172 (S172C), replacing a serine by a cysteine residue. The latter is a conservative amino acid replacement, located in the fourth transmembrane domain of the protein, which follows the second intracellular loop, where mutation T162I is positioned. The mutation S172C occurs in a remarkably conserved position in reptiles, as most of the species with *Mc1r* sequences available to date share a serine residue whereas *L. l. iberica* and *L. l. lepida* have a derived cysteine residue (Fig. 4).

Besides nonsynonymous mutations, three other synonymous changes at positions 351, 411 and 477 were also fixed in European subspecies, separating *L. l. nevadensis* from the other two subspecies (Fig. 5). All five mutations segregate in complete linkage disequilibrium ($N = 46$, Fisher's exact test, $P < 0.001$ with Bonferroni correction). However, *L. l. lepida* and *L. l. iberica* cannot be distinguished from each other by any fixed mutation. Although six haplotypes were found among these two subspecies, five have low frequencies (3–15%) and most of them differ by one synonymous mutation alone from the most frequent haplotype. By contrast, *Lacerta pater* (from Tunisia, Africa) showed a high number of haplotypes, especially considering the small sample size (four lizards), and all haplotypes were unique to the species (Fig. 5). Finally, the main haplotype in *Lacerta tangitana* (from Morocco) differs from *L. l. lepida* by five mutations, including the S172C mutation. However, one heterozygous *L. tangitana* lizard was detected with the most common haplotype of *L. l. lepida* and another allele differing from the previous one by a single mutation (Fig. 5). With the exception of this sample, the cysteine residue in mutation S172C (site 514) was exclusively found in Europe, in both *L. l. lepida* and *L. l. iberica* subspecies, where it was fixed in our sample.

No signature of selection was detected with the McDonald–Kreitman test. For Tajima's *D* test, values were slightly positive for all ocellated lizard species or

Table 1 Number of alleles, haplotypes, segregating (Seg.) sites, synonymous (Syn.) and nonsynonymous (Nonsyn.) substitutions in each ocellated lizard species and subspecies. Haplotype diversity (H), nucleotide diversity (π) and Tajimas's *D* test values are also presented.

Species	No. of alleles	No. of haplotypes	*H*	Seg. sites	Syn. sub	Nonsyn. sub	π	Tajima's *D*
Lacerta lepida	46	10	0.748	13	11	2	0.00656	
L. l. iberica	8	2	0.429	1	1	0	0.00095	0.33350
L. l. lepida	26	5	0.594	4	4	0	0.00165	–0.78167
L. l. nevadensis	12	4	0.742	4	4	0	0.00333	0.46585
Lacerta pater	6	5	0.933	6	5	1	0.00637	0.52043
Lacerta tangitana	8	3	0.464	6	4	2	0.00532	0.15875

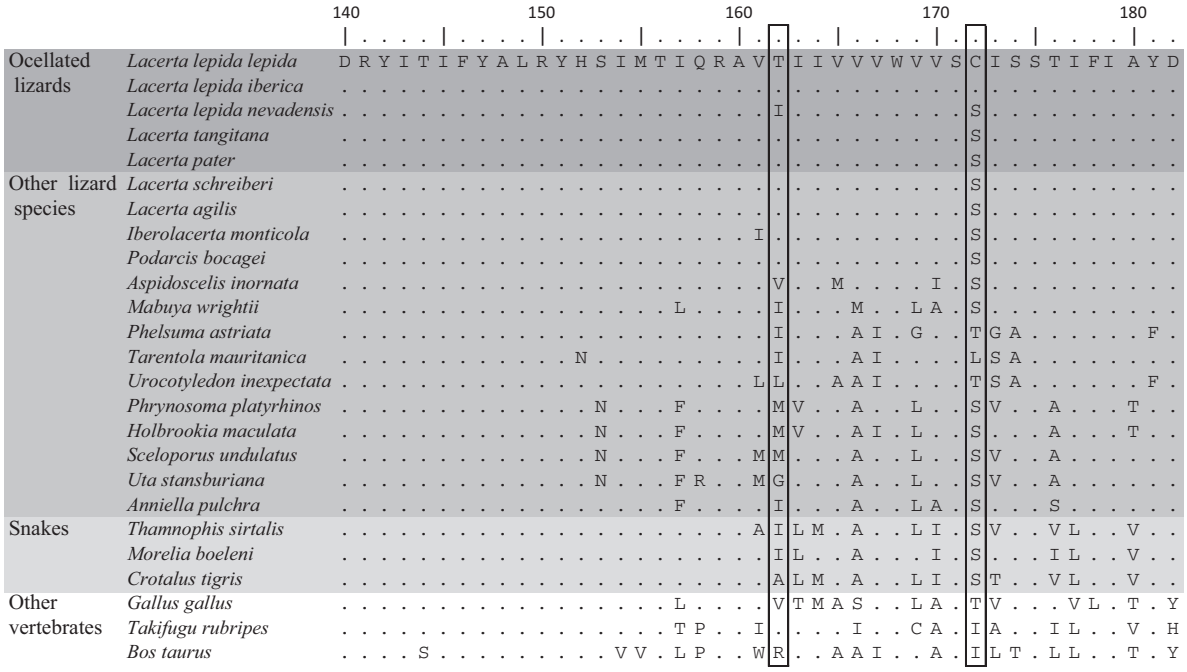

Fig. 4 Partial alignment of *Melanocortin-1 receptor* amino acid chain from ocellated lizards (*Lacerta lepida*, *L. tangitana* and *L. pater*) with other vertebrates, mainly reptile species. The positions where amino acid changes were detected in *L. lepida* are highlighted with boxes (positions 162 and 172).

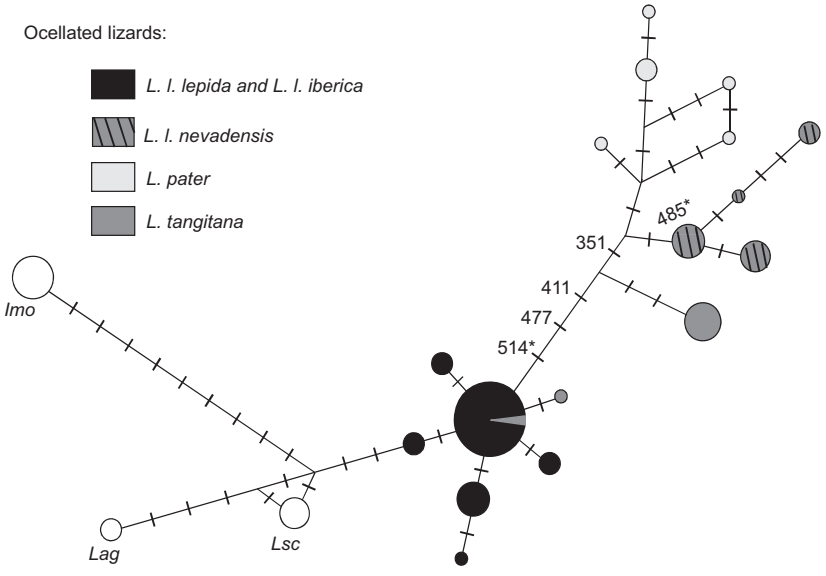

Fig. 5 Minimum spanning haplotype network for the *Melanocortin-1 receptor* gene from 37 lizards. The size of the circles is proportional to sample size. Each mutation between haplotypes is represented by a dash. Site number is indicated for mutations that are fixed for *Lacerta lepida nevadensis* and for *L. l. lepida* and *L. l. iberica* phenotypes. Asterisks denote nonsynonymous changes. Nonocellated lizard haplotypes are represented in white: *L. agilis* (*Lag*), *Iberolacerta monticola* (*Imo*) and *L. shreiberi* (*Lsc*).

subspecies, except for *L. l. lepida* (Table 1), but no value was significantly different from zero.

Association of *Mc1r* with phenotype

Association tests were conducted between *Mc1r* and European ocellated lizard phenotypes. As *L. l. iberica* only differs from *L. l. lepida* phenotypically in the relative proportion of black scales and no fixed mutations in *Mc1r* were found between them, phenotypes were grouped as '*Nev*' (*L. l. nevadensis*) or '*non-Nev*' (*L. l. iberica* and *L. l. lepida*) for association tests. Only three *Mc1r* SNPs were considered, corresponding to the nonsynonymous mutations at sites 485 (T162I) and 514

(S172C), as well as one closely located synonymous SNP at site 477, in linkage disequilibrium with the previous ones. Samples of *L. l. nevadensis* (*Nev*) shared the homozygous genotype for the three SNPs, AA (477)-TT (485)-AA (514), whereas the remaining *L. lepida* samples (non-*Nev*) exhibited the alternative homozygous genotype, GG-CC-TT (Fig. 1), leading to a significant association of phenotypes with *Mc1r* genotype ($N = 160$, Fisher's exact test, $P = 2.2e^{-16}$). Despite the extensive sampling near the contact zone between *L. l. lepida* and *L. l. nevadensis* (Fig. 1b), only two heterozygous individuals were detected, one from population L5 and another from N4. The forward and reverse sequences of these individuals confirmed the heterozygote state at the five polymorphic positions found to be fixed in each subspecies (accession numbers JF732967 and JF732968). The allelic phases for these two hybrids most likely correspond to a haplotype from *L. l. lepida* and another from *L. l. nevadensis*. Inferences about the dominance effect of each allele on *L. l. nevadensis* or *L. l. lepida* phenotypes were not possible due to lack of detailed information on colour traits for individuals sampled across the contact zone.

Discussion

The three subspecies of ocellated lizards with parapatric distributions in the Iberian Peninsula exhibit clear differences in melanin-based colour content and distribution over the lizards' dorsum. The degree of subspecific differences in colour is consistent with the level of genetic differentiation measured by mitochondrial DNA, with an estimated divergence time of 9.43 million years (My) for *L. l. nevadensis* but only around 2 My for *L. l. iberica* (Paulo *et al.*, 2008; Miraldo *et al.*, 2011). The most divergent colour phenotype corresponds to *L. l. nevadensis*, with the prevalence of brown over black scales and a clear reduction in green colour content over the dorsal pattern. In the same way, variation at the candidate gene *Mc1r* is in accordance with the higher level of morphological divergence of *L. l. nevadensis*, as compared to the other Iberian subspecies, which are indistinguishable from each other in the *Mc1r* amino acid chain. A derived and nonconservative amino acid change, T162I, was perfectly associated with the *L. l. nevadensis* phenotype in our sample, with an isoleucine residue fixed at this position. A second amino acid change, S172C, segregates in linkage disequilibrium with the previous one, but the serine residue that occupies this position in *L. l. nevadensis* is shared with all other reptiles investigated to date, except for *L. l. lepida* and *L. l. iberica* that have a cysteine residue instead. Therefore, whereas for mutation T162I the isoleucine is associated with the prevalence of brown scales (putatively due to a partial loss of function of the *Mc1r* receptor), in mutation S172C the cysteine residue seems to be associated with a higher melanization (a putative gain of function), as registered in *L. l. lepida* and *L. l. iberica* phenotypes. In northern Africa, only one lizard from *L. tangitana* has a cysteine residue at mutation S172C, but information on this species' colour variation is scarce and unclear, because previous studies focused on pattern rather than colour variability (Mateo, 1988, 1990; Mateo *et al.*, 1996).

No evidence for positive selection was detected in the present *Mc1r* data set. However, the opportunity for detection of selection with the present tests was slim in such short sequences, with only two amino acid changes (see Hughes, 2007). Nevertheless, it has been previously shown that a single amino acid substitution can have a dramatic effect on phenotype and thus have significant adaptive consequences (Hoekstra *et al.*, 2006). The derived haplotype of *L. l. nevadensis* with a isoleucine residue in mutation T162I is a nonconservative change and is exactly the same amino acid change found in association with the blanched phenotype of little striped whiptail lizard, *Aspidoscelis inornata* (mutation T170I), resulting in a partial loss of function (Rosenblum *et al.*, 2010). Although *A. inornata* is not closely related to ocellated lizards, if future functional studies can confirm T162I as a partial loss-of-function mutation in *L. l. nevadensis*, this might represent another convergence example in *Mc1r* evolution. Both mutations are located in the fourth transmembrane domain of the *Mc1r* protein, and implications of such an amino acid replacement for the physical properties of the protein in *L. l. nevadensis* may be similar to the functional consequences seen in *A. inornata*. Some human variants of *Mc1r* associated with red hair and fair skin that result in diminished function of the protein also have mutations that are homologous or in the immediate vicinity of T162I mutation in ocellated lizards (R160W, R162P and R163Q, homologous to amino acid positions 159, 161 and 162 in the *L. lepida Mc1r* sequence, respectively) (Garcia-Borron *et al.*, 2005). Another example is the cavefish *Aystyanax mexicanus*, where mutation R164C (homologous to residue 159 in *L. lepida*) was shown to be functionally responsible for a brown derived phenotype in cave populations (Gross *et al.*, 2009).

Variation at *Mc1r* sequences indicates low gene flow between *L. l. lepida* and *L. l. nevadensis*, as suggested by the presence of five diagnostic mutations, fixed for all haplotypes detected in each subspecies (including the nonsynonymous mutations referred to above) and by the detection of an extremely low number of hybrids across the contact zone. The phenotype of *L. l. nevadensis* seems to have camouflage advantages in the arid landscapes of the south-eastern Iberian Peninsula. However, neither the causal relationship between *Mc1r* variants and phenotype nor the relative contribution of genetic drift and selection in the evolution of the observed colour pattern can be assessed without further investigations into association in segregating populations or into the fitness and functional effects of genes underlying such colour traits.

A long history of divergence between *L. l. nevandensis* and *L. l. lepida* (Paulo *et al.*, 2008; Miraldo *et al.*, 2011), by the combined effect of selection and genetic drift, helps to explain why such a low number of hybrids were detected in the current contact zone. The two hybrids detected in the present study were sampled at the nearest locations to the subspecies' distribution limits (N4 and L5), located just about 25 km apart. Both hybrids inherited mitochondrial haplotypes from the subspecies of their population of origin, on each side of the contact zone, meaning that hybridization probably resulted from copulation of local females by immigrant males. As ocellated lizard males are territorial, even when young (Castilla, 1989), lower-ranking males may be forced to disperse and seek suboptimal territories, as the best ones are already occupied by older and larger males (Paulo, 1988). For populations closer to the contact zone, male-biased dispersal may increase the opportunity for hybridization, but if their different phenotypes have fitness implications, it may prevent further blending of genomes from the two lineages. Furthermore, slight differences in intraspecific signals between subspecies may promote assortative mating, therefore reducing the opportunity for gene flow between *L. l. lepida* and *L. l. nevadensis*. An alternative hypothesis for the predominance of the brown/grey colour in *L. l. nevadensis* would be the effect on colour variation of genes underlying other traits, in which case the fixed differences in colour between the subspecies would be a by-product not a result of direct selection.

The second amino acid replacement, S172C, found in association with the *L. l. lepida* and *L. l. iberica* phenotype of increased melanin content, corresponds to a derived but conservative change and therefore is less likely to have functional implications. Nevertheless, it is located in a remarkably conserved position among reptiles and belongs to the second intracellular loop of the *Mc1r* protein, a region that seems to have an important role in proper coupling, normal processing and intracellular traffic according to results from functional studies on *Mc1r* human variants (Garcia-Borron *et al.*, 2005). Only functional assays can confirm or reject the functional consequences of the S172C replacement. The involvement of other genes in the melanin-based phenotype in ocellated lizards must also be investigated. The lack of association of the *L. l. iberica* phenotype with *Mc1r* variants suggests that increased melanization in this subspecies as compared to the nominal subspecies may result from regulatory mutations or missed structural mutations in *Mc1r* extremities that were not sequenced in this study or in other genes with important functions in pigmentation. Studies in beach mice have shown that the differences in coat colour result mainly from the interaction of two pigmentation genes, *Mc1r* and *Agouti* (Steiner *et al.*, 2007), whereas melanism in deer mice is associated with mutations in *Agouti* but not in *Mc1r* (Kingsley *et al.*, 2009). Likewise, investigations into convergent coat colour polymorphism in gophers and in some populations from rock pocket mice failed to detect associations with *Mc1r* variation (Hoekstra & Nachman, 2003; Wlasiuk & Nachman, 2007), whereas in Soay sheep, light and dark coat colour are associated with mutations in *Tyrp1*, another gene involved in the melanin synthesis pathway (Gratten *et al.*, 2007).

Finally, melanin-based coloration can only explain variation in the complex colour pattern of ocellated lizards to a limited extent. The genetic mechanisms underlying the formation and distribution of other pigment cells responsible for nonmelanin pigments and structural colours in vertebrates, like xanthophores and iridophores, and how they interfere with melanin-based colours in the overall colour pattern remain largely unknown (Hubbard *et al.*, 2010). Further research on the molecular basis of each colour pigment type is much needed to fully address questions related to diversification and evolution of adaptive colour phenotypes in nonmammalian vertebrates.

Acknowledgments

This research was supported by doctoral scholarships from FCT to Nunes VL (SFRH/BD/21306/2005) and to Miraldo A (SFRH/BD/1696/2004) and by the FCT project POCI/BIA/59288/2004 'Process of speciation: accessing the genic view' coordinated by Paulo OS. All samples were collected under appropriate licences in each country. We thank Juan Pablo de la Vega, Luis Garcia, Gabriel Marin, Eugenia Zarza-Franco and Brent Emerson for helping with fieldwork and two anonymous reviewers for their comments on a previous version of this manuscript.

References

Bandelt, H.-J., Forster, P. & Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **16**: 37–48.

Castilla, A.M. 1989. *Autoecología del lagarto ocelado (Lacerta lepida)*. PhD thesis, Universidad Autónoma de Madrid, Madrid.

Fisher, R.A. 1935. The logic of inductive inference. *J. R. Stat. Soc. Ser. A* **98**: 39–54.

Font, E., Pérez I De Lanuza, G. & Sampedro, C. 2009. Ultraviolet reflectance and cryptic sexual dichromatism in the ocellated lizard, *Lacerta (Timon) lepida* (Squamata: Lacertidae). *Biol. J. Linn. Soc.* **97**: 766–780.

Garcia-Borron, J.C., Sanchez-Laorden, B.L. & Jimenez-Cervantes, C. 2005. Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res.* **18**: 393–410.

Gompel, N. & Prud'homme, B. 2009. The causes of repeated genetic evolution. *Dev. Biol.* **332**: 36–47.

Gratten, J., Beraldi, D., Lowder, B.V., McRae, A.F., Visscher, P.M., Pemberton, J.M. *et al.* 2007. Compelling evidence that a single nucleotide substitution in *TYRP1* is responsible for coat-colour polymorphism in a free-living population of Soay sheep. *Proc. R. Soc. B Biol. Sci.* **274**: 619–626.

Grether, G.F., Kolluru, G.R. & Nersissian, K. 2004. Individual colour patches as multicomponent signals. *Biol. Rev.* **79**: 583–610.

Gross, J.B., Borowsky, R. & Tabin, C.J. 2009. A novel role for Mc1r in the parallel evolution of depigmentation in independent populations of the cavefish *Astyanax mexicanus*. *PLoS Gen.* **5**: e1000326.

Hodar, J.A., Campos, F. & Rosales, B.A. 1996. Trophic ecology of the ocellated lizard *Lacerta lepida* in an arid zone of southern Spain: relationships with availability and daily activity of prey. *J. Arid Environ.* **33**: 95–107.

Hoekstra, H.E. 2006. Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity* **97**: 222–234.

Hoekstra, H.E. & Nachman, M. 2003. Different genes underlie adaptive melanism in different populations of rock pocket mice. *Mol. Ecol.* **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.

Hubbard, J.K., Uy, J.A.C., Hauber, M.E., Hoekstra, H.E. & Safran, R.J. 2010. Vertebrate pigmentation: from underlying genes to adaptive function. *Trends Genet.* **26**: 231–239.

Hughes, A.L. 2007. Looking for Darwin in all the wrong places: the misguided quest for positive selection at the nucleotide sequence level. *Heredity* **99**: 364–373.

Kingsley, E.P., Manceau, M., Wiley, C.D. & Hoekstra, H.E. 2009. Melanism in *Peromyscus* is caused by independent mutations in Agouti. *PLoS ONE* **4**: e6435.

Librado, P. & Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452.

Manceau, M., Domingues, V.S., Linnen, C.R., Rosenblum, E.B. & Hoekstra, H.E. 2010. Convergence in pigmentation at multiple levels: mutations, genes and function. *Philos. Trans. R. Soc. B* **365**: 2439–2450.

Mateo, J.A. 1988. *Estudio sistemático y zoogeográfico de los lagartos ocelados, Lacerta lepida (Daudin, 1802) y Lacerta pater (Lataste 1880) (Sauria, Lacertidae)*. PhD thesis, University of Sevilla, Sevilla.

Mateo, J.A. 1990. Taxonomy and evolution of the North African ocellated lizard, *Lacerta pater* (Lataste, 1880) (Sauria: Lacertidae). *Bonn. Zool. Beitr.* **41**: 203–212.

Mateo, J.A. & Castroviejo, J. 1990. Variation morphologique et révision taxonomique de l'espèce *Lacerta lepida* Daudin, 1802 (Sauria, Lacertidae). *Bull. Mus. Natl. Hist. Nat. Paris* **12**: 691–706.

Mateo, J.A. & López-Jurado, L.F. 1994. Variaciones en el color de los lagartos ocelados; aproximación a la dstribución de *Lacerta lepida nevadensis* Buchholz 1963. *Rev. Esp. Herpetol.* **8**: 29–35.

Mateo, J.A., Lopez-Jurado, L.F. & Guillaume, C.P. 1996. Proteic and morphological variations in ocellated lizards (Lacertidae): a complex of species across the Strait of Gibraltar. *C. R. Acad. Sci. III* **319**: 737–746.

Mateo, J.A., Lopez-Jurado, L.F., Cano, J. & Guillaume, C.P. 1999. Intraspecific variability in nucleolar organiser position in *Lacerta (Timon) lepida*. *Amphib. Reptil.* **20**: 73–77.

Mayer, W. & Bischoff, W. 1996. Beiträge zur taxonomischen revision der gattung *Lacerta* (Reptilia: Lacertidae) teil 1: Zootoca, Omanosaura, Timon und Teira als eigenständige gattungen. *Salamandra* **32**: 163–170.

McDonald, J.H. & Kreitman, M. 1991. Adaptive protein evolution at the Adh locus in Drosophila. *Nature* **351**: 652–654.

Miraldo, A. 2009. *Phylogeography and population dynamics of secondary contact zones of Lacerta lepida in the Iberian Peninsula*. PhD thesis, University of East Anglia, Norwich.

Miraldo, A., Hewitt, G.M., Paulo, O.S. & Emerson, B.C. 2011. Phylogeography and demographic history of *Lacerta lepida* in the Iberian Peninsula: multiple refugia, range expansions and secondary contact zones. *BMC Evol. Biol.* **11**: 170.

Moreira, P.L., Nunes, V.L., Martín, J. & Paulo, O.S. 2007. Copulatory plugs do not assure high first male fertilisation success: sperm displacement in a lizard. *Behav. Ecol. Sociobiol.* **62**: 281–288.

Mundy, N.I. 2005. A window on the genetics of evolution: *Mc1r* and plumage colouration in birds. *Proc. R. Soc. B Biol. Sci.* **272**: 1633–1640.

Paulo, O.S. 1988. *Estudo eco-etológico da população de Lacerta lepida (Daudin 1802) (Sauria, Lacertidae) da ilha da Berlenga*. Faculdade de Ciências da Universidade de Lisboa, Lisboa.

Paulo, O.S., Dias, C., Bruford, M.W., Jordan, W.C. & Nichols, R.A. 2001. The persistence of pliocene populations through the pleistocene climatic cycles: evidence from the phylogeography of an Iberian lizard. *Proc. R. Soc. Lond. B Biol. Sci.* **268**: 1625–1630.

Paulo, O.S., Pinheiro, J., Miraldo, A., Bruford, M.W., Jordan, W.C. & Nichols, R.A. 2008. The role of vicariance vs. dispersal in shaping genetic patterns in ocellated lizard species in the western Mediterranean. *Mol. Ecol.* **17**: 1535–1551.

Pina-Martins, F. & Paulo, O.S. 2008. Concatenator: sequence data matrices handling made easy. *Mol. Ecol. Resour.* **8**: 1254–1255.

Protas, M.E. & Patel, N.H. 2008. Evolution of coloration patterns. *Ann. Rev. Cell Dev. Biol.* **24**: 425–446.

Raia, P., Guarino, F.M., Turano, M., Polese, G., Rippa, D., Carotenuto, F. *et al.* 2010. The blue lizard spandrel and the island syndrome. *BMC Evol. Biol.* **10**: 289.

Rosenblum, E.B., Hoekstra, H.E. & Nachman, M.W. 2004. Adaptive reptile color variation and the evolution of the Mc1r gene. *Evolution* **58**: 1794–1808.

Rosenblum, E.B., Römpler, H., Schöneberg, T. & Hoekstra, H.E. 2010. Molecular and functional basis of phenotypic convergence in white lizards at White Sands. *Proc. Natl Acad. Sci. USA* **107**: 2113–2117.

Roulin, A. 2004. The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. *Biol. Rev.* **79**: 815–848.

Steiner, C.C., Weber, J.N. & Hoekstra, H.E. 2007. Adaptive variation in beach mice produced by two interacting pigmentation genes. *PLoS Biol.* **5**: 1880–1889.

Stephens, M. & Scheet, P. 2005. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. *Am. J. Hum. Genet.* **76**: 449–462.

Stephens, M., Smith, N.J. & Donnelly, P. 2001. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.* **68**: 978–989.

Tajima, F. 1989. Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585–595.

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 Res.* **22**: 4673–4680.

Wlasiuk, G. & Nachman, M.W. 2007. The genetics of adaptive coat color in gophers: coding variation at *Mc1r* is not responsible for dorsal color differences. *J. Hered.* **98**: 567–574.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Dorsal colour pattern of European ocellated lizard subspecies.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 6 May 2011; revised 22 June 2011; accepted 23 June 2011