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GENETIC ARCHITECTURE, EVOLUTION AND MAINTENANCE OF A COLOR CLINE AT A CONTINENTAL SCALE

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Département d'Ecologie et d'Evolution

GENETIC ARCHITECTURE, EVOLUTION AND MAINTENANCE OF A COLOR CLINE AT A CONTINENTAL SCALE

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Sylvain ANTONIAZZA

Master de l'Université de Lausanne

Jury

Prof. Nicolas Mermod, Président
Prof. Jérôme Goudet, Directeur de thèse
Prof. Alexandre Roulin, Directeur de thèse
Prof. Oscar Eduardo Gaggiotti, expert
Prof. Tadeusz Kawecki, expert

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Prof. Nicolas Mermod

UNIL | Université de Lausanne
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“In many cases at least, the species will prove to consist of a population showing adaptive clines running in various directions: the continuous gradation will be broken up by various forms of isolation, which, by impeding interbreeding and the free flow of genes, will accentuate the mean adaptive differences between adjacent groups, as well as in some cases introducing non-adaptive differences.”

Julian Huxley, 1938

Contents

Summary

On a geological time scale the conditions on earth are very variable and biological patterns (for example the distributions of species) are very dynamic. Understanding large scale patterns of variation observed today thus requires a deep understanding of the historical factors that drove their evolution.

In this thesis, we reevaluated the evolution and maintenance of a continental color cline observed in the European barn owl (Tyto alba) using population genetic tools. The colour cline spans from south-est Europe where most individual have pure white underparts to north and east Europe where most individuals have rufous-brown underparts. Our results globally showed that the old scenario, stipulating that the color cline evolved by secondary contact of two color morphs (white and rufous) that evolved in allopatry during the last ice age has to be revised.

We collected samples of about 700 barn owls from the Western Palearctic to establish the first population genetic data set for this species. Individuals were genotyped at 22 microsatellites markers, at one mitochondrial gene, and at a candidate color gene. The color of each individuals was assessed and their sex determined by molecular methods.

We first showed that the genetic variation in Western Europe is very limited compared to the heritable color variation. We found no evidences of different glacial lineages, and showed that selection must be involved in the maintenance of the color cline (chapter 1). Using computer simulations, we demonstrated that the post-glacial colonization of Europe occurred from the Iberian Peninsula and that the color cline could not have evolved by neutral demographic processes during this colonization (chapter 2). Finally we reevaluated the whole history of the establishment of the Western Palearctic variation of the barn owl (chapter 3): This study showed that all Western European barn owls descend

from white barn owls phenotypes from the Middle East that colonized the Iberian Peninsula via North-Africa. Following the end of the last ice age (20'000 years ago), these white barn owls colonized Western Europe and under selection a novel rufous phenotype evolved (during or after the colonization). An important part of the color variation could be explained by a single mutation in the melanocortin-1-receptor (MC1R) gene that appeared during or after the colonization. The colonization of Europe reached until Greece, where the rufous birds encountered white ones (which reached Greece from the Middle East over the Bosporus) in a secondary contact zone. Our analyses show that white and rufous barn owls in Greece interbreed only to a limited extent. This suggests that barn owls are at the verge of becoming two species in Greece and demonstrates that European barn owls represent an incipient ring species around the Mediterranean.

The revisited history of the establishment of the European barn owl color cline makes this model system remarkable for several aspects. It is a very clear example of strong local adaptation that can be achieved despite high gene flow (strong color and MC1R differentiation despite almost no neutral genetic differentiation). It also offers a wonderful model system to study the interactions between colonization processes and selection processes which have, for now, been remarkably understudied despite their potentially ubiquitous importance. Finally it represents a very interesting case in the speciation continuum and appeals for further studying the amount of gene flow that occurs between the color morphs in Greece.

Résumé

Sur l'échelle des temps géologiques, les conditions sur terre sont très variables et les patrons biologiques (telle que la distribution des espèces) sont très dynamiques. Si l'on veut comprendre des patrons que l'on peut observer à large échelle aujourd'hui, il est nécessaire de d'abord comprendre les facteurs historiques qui ont gouverné leur établissement.

Dans cette thèse, nous allons réévaluer, grâce à des outils modernes de génétique des populations, l'évolution et la maintenance d'un cline de couleur continental observé chez l'effraie des clochers européenne (Tyto alba). Globalement, nos résultats montrent que le scenario accepté jusqu'à maintenant, qui stipule que le cline de couleur a évolué à partir du contact secondaire de deux morphes de couleur (blanches et rousses) ayant évolué en allopatrie durant les dernières glaciations, est à revoir.

Afin de constituer le premier jeu de données de génétique des populations pour cette espèce, nous avons récolté des échantillons d'environ 700 effraies de l'ouest Paléarctique. Nous avons génotypé tous les individus à 22 loci microsatellites, sur un gène mitochondrial et sur un autre gène participant au déterminisme de la couleur. Nous avons aussi mesuré la couleur de tous les individus et déterminé leur sexe génétiquement.

Nous avons tout d'abord pu montrer que la variation génétique neutre est négligeable en comparaison avec la variation héritable de couleur, qu'il n'existe qu'une seule lignée européenne et que de la sélection doit être impliquée dans le maintien du cline de couleur (chapitre 1). Grâce à des simulations informatiques, nous avons démontré que l'ensemble de l'Europe de l'ouest a été recolonisé depuis la Péninsule Ibérique après les dernières glaciations et que le cline de couleur ne peut pas avoir évolué par des processus neutre durant cette colonisation (chapitre 2). Finalement, nous avons réévalué l'ensemble de l'histoire postglaciaire de l'espèce dans l'ouest Paléarctique (chapitre 3): l'ensemble des

effraies du Paléarctique descendent d'effraie claire du Moyen-Orient qui ont colonisé la péninsule ibérique en passant par l'Afrique du nord. Après la fin de la dernière glaciation (il y a 20'000 ans), ces effraies claires ont colonisé l'Europe de l'ouest et ont évolués par sélection le phénotype roux (durant ou après la colonisation). Une part importante de la variation de couleur peut être expliquée par une mutation sur le gène MC1R qui est apparue durant ou juste après la colonisation. Cette vague de colonisation s'est poursuivie jusqu'en Grèce où ces effraies rousses ont rencontré dans une zone de contact secondaire des effraies claires (qui sont remontées en Grèce depuis le Moyen-Orient via le Bosphore). Nos analyses montrent que le flux de gènes entre effraies blanches et rousses est limité en Grèce, ce qui suggère qu'elles sont en passe de former deux espèces et ce qui montre que les effraies constituent un exemple naissant de spéciation en anneaux autour de la Méditerranée.

L'histoire revisitée des effraies des clochers de l'ouest Paléarctique en fait un système modèle remarquable pour plusieurs aspects. C'est un exemple très claire de forte adaptation locale maintenue malgré un fort flux de gènes (différenciation forte de couleur et sur le gène MC1R malgré presque aucune structure neutre). Il offre également un très bon système pour étudier l'interaction entre colonisation et sélection, un thème ayant été remarquablement peu étudié malgré son importance. Et il offre finalement un cas très intéressant dans le « continuum de spéciation » et il serait très intéressant d'étudier plus en détail l'importance du flux de gènes entre les morphes de couleur en Grèce.

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General introduction

The starting point of my work on the barn owl (*Tyto alba*) was to revisit with modern tools the old known pattern of colour variation of this species in Europe (Roulin 2003; Voous 1950). The European barn owl presents a colour gradient from south-west Europe where most individuals have pure white underparts to northeast Europe where most individuals present rufous-brown underparts. The main aim was to test whether the gradient, or cline, in colour was the result of the adaptation of the barn owl to an environmental gradient or could result from other processes. I used molecular genetic tools to infer ecological characteristics of the species and thus was able to study the colour variation in a well described ecological and historical context.

I will first explain a few important concepts for the understanding of the major aims of this thesis: local adaptation, which is the adaptation of different populations to different ecological conditions; second, the history of the concept of cline, which is simply a technical term that denotes a gradient of any character; and third the history of the last glaciation which have shaped the genetic variation of many species in Europe. I will then present in more detail the European barn owl as a model species and finally give an outlook of the present thesis.

Local adaptation: is selection strong enough to generate local differences between populations?

Variations at the intraspecific level have intrigued biologists for a long time and constituted the raw material of many discussions and theories. The first two chapters of the

"origin of the species", the book that made the foundation of all the modern biology, is actually about the variation in domestic and wild organisms respectively (Darwin 1859). If Darwin discussed mostly the intraspecific variation within a single population, the description of intraspecific variation in a geographical context has also a long history. For example, the Bergmann's rule describes the frequent observation that many animals present bigger body sizes at high latitudes than closer to the equator and is a classic ecogeographic rule (see discussion on Bergmann's rule in Lomolino *et al.* 2006).

Many of these variations described by naturalists are generally assumed to be adaptive and it is only in the last few decades that the "local adaptation" concept has been build up to rigorously analyse and test those cases (Kawecki & Ebert 2004). Evidences for local adaptation in space can be found in many different contexts, including host-pathogen interactions (Greischar & Koskella 2007; Kaltz & Shykoff 1998; Sotka 2005), selection linked to climate and temperature (McKay *et al.* 2001; Savolainen *et al.* 2007), altitude (Byars *et al.* 2007; Keller *et al.* 2013; Samietz *et al.* 2005; Storz & Dubach 2004), water salinity (Gomez-Mestre & Tejedo 2003; McCairns & Bernatchez 2008), interspecific competition (Abjornsson *et al.* 2004; Grondahl & Ehlers 2008), and camouflage (Hoekstra *et al.* 2005). Examples will probably continue to accumulate in the literature and time has probably come for a review or a book about the subject (but see Schluter 2000 for a first attempt that would deserve deeper treatment).

The local adaptation concept has also become very important in the last two decades in the speciation literature with the growing importance of the so-called "ecological speciation" (Nosil 2012). The link between adaptation and speciation was neglected for a long time and it is only since the beginning of the 80's that this issue has started to be studied in depth (Coyne & Orr 2004). This discussion goes along with a vivid debate on the

relative importance of selection and drift in the process of speciation (Coyne & Orr 2004). The dominant view about the necessity of the reduction of gene flow between population of an incipient species by some form of allopatry advocated for example by Ernst Mayr (1963) has been reevaluated during the last few decades. Nowadays, a more balanced view on speciation has started to emerge (Feder *et al.* 2013; Fitzpatrick *et al.* 2009; Mallet *et al.* 2009).

A more integrated view of the process of speciation is thus needed with a comprehensive view on history, demography and ecology. We will see in this thesis, why I think that the European barn owl provides an appropriate model system to study the interplay between historical factors, local adaptation and ultimately speciation.

Cline: the evolution of a technical term for "gradient"

The term "cline" was originally coined by Julian Huxley in 1938 to describe a "gradation in measurable characters" (Huxley 1938). Huxley first proposed it as "an auxiliary taxonomic principle" by insisting on the fact that species do not always consist of homogenous units but sometimes also present gradient of variation at the intraspecific level. The early population geneticists quickly realised that a cline might result from an equilibrium between selection and gene flow and that quantitative estimates of the two forces could well be derived from clinal patterns of variation (early attempt include Fisher 1950; Haldane 1948). One next important milestone was passed with the seminal works of John Endler (Endler 1977; Endler 1973). He developed complementary studies using theoretical developments, computer simulations and experimental cline with *Drosophila* to investigate the evolution and maintenance of clines. Endler first emphasised that clines might result

from very different processes that do not necessarily require selection: isolation by distance, secondary contact of previously allopatric populations, secondary contact between two locally adapted populations at an ecotone or adaptation to a continuous environmental gradient. Disentangling those origins of clines is still a major challenge in the description of any clinal pattern, including the one observed in the barn owl. John Endler was also a precursor of many modern questions regarding speciation by proposing that differentiation and ultimately parapatric speciation along cline driven by local adaptation despite the presence of gene flow might be an important process in evolution (Endler 1977).

Many of these early interests in clines are still fruitful area of research today and have evolved with the tremendous technical development of modern biology. One good example is the development of the early theoretical treatment of cline to infer populations' genetic parameters like selection. This was particularly fruitful in the context of hybrid zones (Barton & Gale 1990; Barton & Hewitt 1985). Those methods are still widely used and developments of tools is still important (eg. Derryberry *et al.* 2013; Gay *et al.* 2008). These approaches permit to estimate population genetics parameters and infer evolutionary processes in secondary contact zones (eg. Gay *et al.* 2009) or in ecotones between locally adapted populations (eg. Mullen & Hoekstra 2008). Some of the early findings still have important and sometimes underestimated consequences like for example the criticism of Bierne et al. (2011) on the study of the genetic basis of local adaptation. Modern development of these technics to use the recently available genomics dataset will probably be a major research venue for the identification of the genetic basis of speciation (e.g. Gompert & Buerkle 2011).

The study of clines is also fruitful in different contexts than in secondary contact settings. The study of clines, with models of parapatric speciation along them, has for

example an importance in the context of the "speciation with gene flow" debate which is one of the important question regarding speciation today (Doebeli & Dieckmann 2003; Lande 1982; Leimar *et al.* 2008). Clines at continental scales are interesting for many aspects including the current human-induced global changes, for example the evolution driven by adaptation to a changing climate (eg. Millien *et al.* 2006; Umina *et al.* 2005) or the evolution of large scale variation following the invasion of a new range (eg. Huey *et al.* 2000; Kooyers & Olsen 2012; Montague *et al.* 2008) and even simply local adaptation (Demont *et al.* 2008; Gockel *et al.* 2001; Hangartner *et al.* 2012; Long & Singh 1995; Palo *et al.* 2003; Savolainen *et al.* 2007; Volis *et al.* 2005).

One last example of modern questions regarding clines might eventually be the revisiting of the establishment of clines by "neutral demographic processes", especially range expansion. The genetic consequences of range expansion are still poorly investigated, but it has been clear for almost a decade that such expansion can generate cline that are very similar to cline generated by selection and that the origin of cline might be more difficult to establish having those process in mind (Excoffier *et al.* 2009).

Despite being an "old topic" in population genetics, the study of cline still have many very useful implications and some questions need definitely still to be answered.

Glaciations: a major determinant of current genetic variation at temperate latitudes

The climatic conditions on earth have always been changing. If we look back in the past, we would see that the last two million years of earth history (Pleistocene and Holocene) have been characterized by strong fluctuations in the climatic conditions between

cold and warmer periods. We are currently in one of those warmer period (independently from global warming), but 20'000 thousands years ago the Alps were under several thousands meters of ice. During this last glacial maximum (called Würm in Europe and Wisconsinian in America), most of western Europe was characterized by a tundra habitat (Flint 1971). It is evident that such dramatic changes have a huge influence on the biogeography of many species (for a very general review at the world scale see Lomolino *et al.* 2006).

Phylogeography is the science of studying genetic lineages in space. This domain was founded in the 80s with the apparition of modern molecular genetic tools (PCR, sequencing, etc.). One of its major successes is the possibility to reconstruct recent history of many taxons by the resolution given by molecular tools. The last glaciation being only a few thousand years back in the past, phylogeography provides a wonderful tool to study the history of many species in the last 20'000 years. One of the major successes of phylogeography was the reconstruction and a better understanding of the fate of many organisms during the last ice age (Avise 2000)..

From many phylogeographic studies on very different organisms inhabiting temperate Europe today, some common patterns emerge. For obvious climatic reasons, many species had to migrate south to track suitable conditions. Many species, that are nowadays spread all over Western Europe, had to retract their rang to the southern peninsula of the continent (Iberia, Italy and/or Balkans). After the maximum of the last ice age (around 20'000 years ago, see Clark *et al.* 2009 for a recent reevalution of the timing of the last glacial maximum), many of those taxa colonized back northern Europe. If the species would have retreated in more than one of the peninsula, we can generally trace back their

glacial origin and find the secondary contact zone where the population coming from the several refugia meet back (Hewitt 1996, 1999, 2000; Taberlet *et al.* 1998).

Given that our model species, the European barn owl is very sensitive to climate (Altwegg *et al.* 2006; Marti & Wagner 1985; Massemin & Handrich 1997), it makes little doubt that during the last glacial maximum this species retreated in some form of refugia in the south of Europe or even in north Africa (see also below).

The European barn owl (*Tyto alba*): a well-known species

The European barn owl is a medium sized nocturnal raptor. It was considered as one of the six cosmopolitan bird species for a long time (e.g. Newton 2003), but recent works seems to indicate that the barn owls from America and Oceania might be different species (König & Weick 2008; Nijman & Aliabadian 2013). With its wide distribution and its antropophilic habits, the barn owl (in a broad sense) has been very intensively studied for a long time. A search for the keyword: "barn owl" on Web of Knowledge® brings for example approximately 5923 results (on 16.06.2014). If most of this literature stems actually from zoological, ecological or behavioural publications, the species is also a model organism for a good corpus of literature in neurosciences (860 of 5923 aforementioned references are in the area "Neurosciences Neurology"). The barn owl is also the subject of many books: a search for "barn owl" on the natural history book shop (www.nhbs.com) on the 16.06.2014 lead to 29 results among which 11 monographs on the species, 9 technical reports mainly from the British Isles and a few more books.

But not from a population genetics point of view

Given the huge amount of literature published and work that has been done on the species, it is surprising that almost no population genetics work have been done before the start of the present thesis (with the exeption of Matics *et al.* 2005). The work of Mátics and his co-author was very preliminary with only two populations, in Hungary and Switzerland. Using RAPD markers, they found that Barn owl genetic diversity is higher in Switzerland than in Hungary. And that male present stronger genetic structure than females and that the genetic structure between Hungary and Switzerland is substantial (ϕ_{ST} = 0.21). These results were compatible with the classical understanding of the evolution of the European colour cline that is presented below.

The classical view on the evolution of the west European colour cline

A first important contribution in the description of the Barn owl European colour cline was published in 1950 by Karel H. Voous (Voous 1950). From 412 museum skins, Voous described the geographic variation in Barn owl colour in a sampling spanning a transect from western north-Africa to north eastern Germany. He then interpreted the observed colour cline as resulting from a secondary contact between two colour morphs that would have evolved in allopatry. He hypothesised the two parent populations to originate from the Mediterranean region for the white barn owls (*Tyto alba alba*) and Crimea or Bulgaria for the dark breasted barn owls (*T. a. guttata*).

A new dataset permitting to refine the description of the European barn owl colour variation was compiled by Alexandre Roulin and published in 2003 (Roulin 2003). The number of individuals sampled was much bigger than the sampling by Voous (1340

individuals), but the geographic extant was similar. There was no reason to doubt the secondary contact hypothesised by Voous from this data set and his interpretations were retaken.

In the only published European population genetics contribution (Matics *et al.* 2005), Mátics interpreted the higher genetic diversity found in Switzerland in comparison to the one of Hungary as compatible with the secondary contact hypothesis. The centre of this secondary contact (Switzerland) could be hypothesis to be more genetically diverse than a region situated further from the hypothesis secondary contact zone (Hungary) and this is what they found in that article.

The classical view of the evolution of the barn owl European colour cline is thus that the colour morphs evolved in allopatry in the Western Mediterranean region and Crimea/Bulgaria for the white and dark morphs respectively. The colour cline would then have evolved by secondary contact after the ice age. This view first described by Voous (Voous 1950) has never been challenged, even with larger sampling (Roulin 2003) or genetic data (Matics *et al.* 2005).

Beside those publications studying and comparing colour variation between populations, some important work has been done on the colour variation within population, especially in a well-studied Swiss population. Several cross-fostering experiments have shown that the colour variation has a strong genetic basis, at least in Switzerland (Roulin & Dijkstra 2003; Roulin *et al.* 1998). Roulin and Dijkstra (2003) estimated the heritability of the colour variation at h^2=0.81. Mating patterns were also investigated and it was shown that the assortment was random in respect to coloration in Switzerland (Roulin 1999), in France (Baudvin 1986) and in Hungary (Matics *et al.* 2002).

The starting point of the present thesis was thus to reappraise the evolution and maintenance of the strongly genetically determined European barn owl colour cline with modern molecular ecological tools.

Aim of the different chapters

In the first chapter of this thesis, we will compare the structure of the colour variation to the neutral genetic structure measured with microsatellite markers for the west European cline (Iberian Peninsula to north of the Balkans). We will use the pattern of isolation by distance on the matrix of pairwise F_{ST} of the microsatellites markers and the matrix of pairwise Q_{ST} of colour to test if the colour variation evolved by a local adaptation process.

In the second chapter, we will test several plausible post-glacial demographic scenarios that might explain the main observed population genetics pattern. Using a spatially explicit Approximate Bayesian Computation framework (ABC), we will build up a demographic model that could well model the genetic variation observed today. This model will then be used to test if neutral genetic simulations might reproduce a pattern such as the one observed for colour and neutral genetic variation of the first chapter.

In the last chapter, the whole western Palearctic distribution has been sampled and we will use this dataset to decipher further the post-glacial history of the European barn owls. Along with the microsatellites markers, we will add a mitochondrial gene for a subsample of the individuals. We will also show data for a candidate gene that seems to explain a good part of the colour variation in Europe. We will show results indicating that the

Western Palearctic barn owls present pattern of a ring species around the Mediterranean basin.

In all the three chapters, we will see that the classical view on the Evolution of the colour variation in two glacial refugia is probably erroneous and that we have to change our perspective on the evolution of the barn owl colour cline. This paradigm shift has strong consequences on how the west European colour cline evolved and opens very interesting questions on the dynamics of adaptation and speciation that we will discuss in the general discussion of the present thesis.

Chapter 1: Local adaptation maintains clinal variation in melanin-based coloration of European barn owls (*Tyto alba*)

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Abstract

Ecological parameters vary in space, and the resulting heterogeneity of selective forces can drive adaptive population divergence. Clinal variation represents a classical model to study the interplay of gene flow and selection in the dynamics of this local adaptation process. Although geographic variation in phenotypic traits in discrete populations could be remainders of past adaptation, maintenance of adaptive clinal variation requires recurrent selection. Clinal variation in genetically determined traits is generally attributed to adaptation of different genotypes to local conditions along an environmental gradient, although it can as well arise from neutral processes. Here, we investigated whether selection accounts for the strong clinal variation observed in a highly heritable pheomelanin-based color trait in the European barn owl by comparing spatial differentiation of color and of neutral genes among populations. Barn owl's coloration varies continuously from white in southwestern Europe to reddish-brown in northeastern Europe. A very low differentiation at neutral genetic markers suggests that substantial gene flow occurs among populations. The persistence of pronounced color differentiation despite this strong gene flow is consistent with the hypothesis that selection is the primary force maintaining color variation among European populations. Therefore, the color cline is most likely the result of local adaptation.

Introduction

The relative role of adaptive versus neutral processes in generating and maintaining genetic and phenotypic variation among as well as within species is still under debate (e.g. Nei 2005; Lynch 2007). Whereas the neutral forces of mutation, drift, and migration result in stochastic allele frequency changes over time and space, natural selection is a directed process eliminating deleterious alleles from populations and carrying advantageous alleles to fixation. If directional selection is the prevailing force of evolution, a paradox emerges: although in the long run natural populations should lose variation at both the phenotypic and functional genetic levels, they usually exhibit high genetic diversity and often extensive phenotypic variation. In homogeneous environments mutation and balancing selection may account for the maintenance of diversity (e.g. Star et al. 2007). However, environments are rarely constant, neither at the spatial nor the temporal scale, and over the evolutionary timescale populations are expected to adapt to the prevailing environmental conditions, that is, to be locally adapted. This selection linked to spatiotemporal environmental heterogeneity may be a major force promoting and maintaining phenotypic and genetic diversity both within and among populations (Felsenstein 1976; Hedrick et al. 1976; Hedrick 1986). Although a potential role of adaptation to temporally fluctuating selection in the maintenance of phenotypic variation has been rarely acknowledge (but see Grant & Grant 2002), local adaptation to spatially varying selection has been widely studied. Among the best-documented sources of local adaptation figure host–pathogen interactions (for reviews see Kaltz & Shykoff 1998; Sotka 2005; Greischar & Koskella 2007). Other examples of local adaptation include selective pressures induced by climate and temperature (McKay et al. 2001; Savolainen et al. 2007), altitude (Storz & Dubach 2004; Samietz et al. 2005; Byars et al. 2007), water salinity (Gomez-Mestre & Tejedo 2003; McCairns and Bernatchez 2008),

interspecies competition and predation (Abjornsson et al. 2004; Grondahl & Ehlers 2008), or soil color-related camouflage against predators (Hoekstra et al. 2005).

Local adaptation may however not be fully achieved, as exchange of individuals adapted to alternative environments may move populations away from the locally optimal phenotype (Postma & van Noordwijk 2005; Räsänen & Hendry 2008). In addition, local adaptation restricts gene flow if the selection gradient between the alternative environments is steep enough to reduce immigrants' probability to reproduce (Nosil et al. 2005). This implies that gene flow can impede local adaptation, whereas inversely local adaptation can lead to reproductive isolation and ultimately speciation (Nosil 2008; Räsänen & Hendry 2008).

Studying the interplay of selection and gene flow thus represents a central issue to the understanding of the evolution of local adaptation and phenotypic diversity (Bridle & Vines 2007). Clines in continuous populations are of particular interest in this context. Although in discrete populations differentiation in fitness-relevant phenotypic traits may reflect historical selection, the persistence of clinal variation requires recurrent spatially heterogeneous selection along environmental gradients to counterbalance the homogenizing effect of gene flow (Haldane 1948; Slatkin 1973). Many important environmental variables vary continually in space, and may lead to strong clinal variation in fitness-related traits (Huey et al. 2000). However, clines can also be an outcome of neutral evolution, being generated by genetic drift in populations connected through spatially limited gene flow (isolation-by-distance) (Endler 1977), by admixture of previously isolated populations (secondary contact) (Slatkin 1973; Barton & Hewitt 1985), or by spatial population expansions (Klopfstein et al. 2006; Excoffier & Ray 2008). Such clines arising as a result of colonization history have been demonstrated recently for phenotypic traits in two

plant species (Keller et al. 2009, see also Vasemägi 2006 for a discussion on clines in gene frequencies in Drosophila). Thus, before invoking selection to explain clinal phenotypic variation, the null hypothesis of neutral evolution should be tested (Gould & Johnston 1972; Storz 2002).

One striking example of clinal phenotypic variation is found in the barn owl (*Tyto alba*) (Roulin et al. 2009). Across all major areas of its almost worldwide distribution, this species displays geographic variation in predominantly genetically determined pheomelanic coloration (Roulin et al. 1998; Roulin & Dijkstra 2003). The color cline is most pronounced on the European continent, where coloration of the body underside continuously varies from white in the southwest to reddish-brown in the northeast (Roulin 2003). Although less work has been carried out on pheomelanic compared to eumelanic color variation in this species, observations suggest that the degree of pheomelanism could be of functional importance. In Central Europe, it is related to diet, breeding rate, and growth rate (Roulin 2004; Roulin and Altwegg 2007; Roulin et al. 2008). However, neutral models of cline evolution have not been tested so far.

The comparison of the levels of population differentiation at putatively selected traits to differentiation at neutral genetic markers allows disentangling adaptive from neutral phenotypic differentiation (for reviews see Merilä & Crnokrak 2001; McKay & Latta 2002; Leinonen et al. 2008; Pujol et al. 2008; Whitlock 2008). If traits evolve neutrally, the proportion of their variation among populations should on average be identical to the proportion of among-population variation in allele frequencies at neutral loci. Q_{ST} represents a metric of population differentiation at a quantitative trait, and under neutrality thus should equal F_{ST}, its analog of genetic differentiation at neutral genetic markers. When Q_{ST} for a given trait exceeds F_{ST}, this indicates that phenotypic differentiation has been

driven by selection for different phenotypes in different populations on the trait under study or on genetically correlated traits (Wright 1951; Spitze 1993). To test whether clinal color variation in the barn owl is maintained by selection on the European continent, we compared the differentiation of coloration and neutral genetic variation among 18 populations.

Methods and Materials

Sampling and color measurements

To measure barn owl coloration across Europe, breast feathers from a total of 373 birds were sampled by collaborators working in survey programs in 18 populations (Fig. 1).

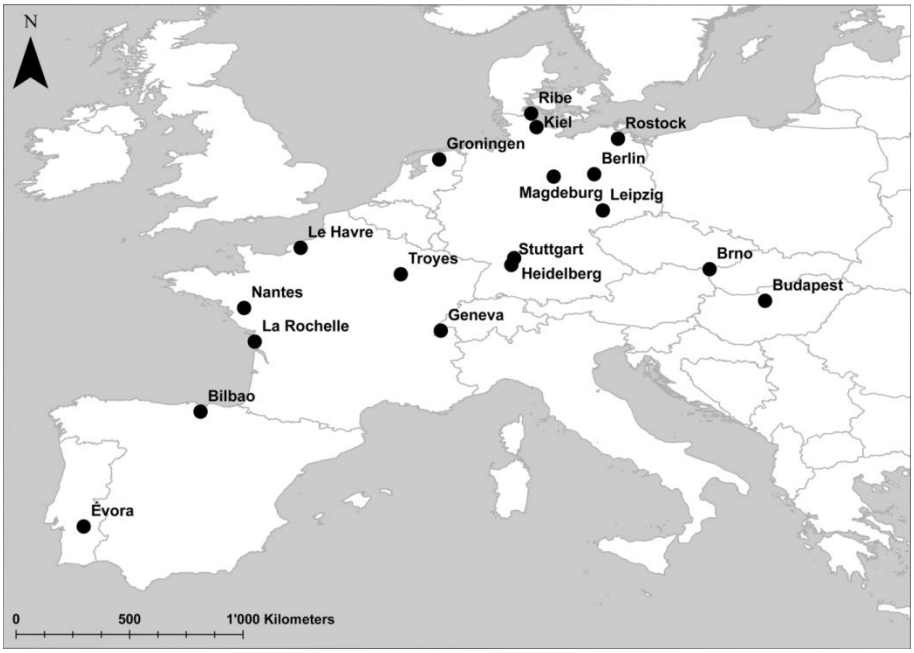

Figure 1. Map showing the population sampling locations. The biggest city in an 80 km radius around the actual sampling area is indicated.

Sampling was conducted during the breeding season 2007, except for two populations in The Netherlands and Hungary (2004–2005). To minimize the probability that

individuals were immigrants from other populations, only nestlings were considered and only one of them was randomly chosen per brood to reduce the probability that individuals are closely related (Table 1, adults were added in two populations with small sample size). The sampling area covered a maximum distance of 2395 km between Évora, Portugal, and Budapest, Hungary (Fig. 1, Table 1). The minimal and mean distances between two populations were 31 km and 864 km (SD: 531 km), respectively.

Table 1. Population samplings' characteristics

Population	Country	Dist. from Évora (km)	N		H_o	H_e	Comments
			F	M			
Évora	P	0	7	14	0.68	0.68	-
Bilbao	E	638	6	5	0.63	0.67	6 juveniles, 5 adults
La Rochelle	F	986	9	3	0.63	0.64	8 juveniles, 5 adults
Nantes	F	1092	10	13	0.65	0.65	-
Le Havre	F	1404	8	7	0.65	0.66	-
Geneva	CH	1444	10	17	0.62	0.65	-
Troyes	F	1500	19	9	0.67	0.65	-
Stuttgart	D	1777	7	13	0.70	0.64	-
Heidelberg	D	1801	9	8	0.71	0.68	-
Groningen	NL	1943	15	14	0.64	0.63	-
Magdeburg	D	2106	15	15	0.59	0.64	-
Leipzig	D	2126	6	13	0.64	0.65	-
Berlin	D	2198	13	14	0.70	0.66	-
Kiel	D	2219	10	10	0.70	0.64	-
Ribe	DK	2253	10	7	0.62	0.65	-
Brno	CZ	2281	9	11	0.68	0.68	-
Rostock	D	2342	14	7	0.64	0.67	-
Budapest	H	2395	9	7	0.71	0.68	-

Pheomelanin-based plumage color of each individual bird was measured from one to five breast feathers (mean: 4.03, SD: 1.10), depending on the number of feathers available. To measure color, reflectance spectra from four points per breast feather were captured with a S2000 spectrophotometer (Ocean Optics, Dunedin, FL) and a dual deuterium and halogen 2000 light source (Mikropackan, Mikropack, Ostfildern, Germany). For each reflectance spectrum, the brown chroma was calculated following Montgomerie (2006). The brown chroma represents the contribution of the red part of the spectrum (600–700 nm) to the complete visible spectrum (300–700 nm). For each individual, the brown chroma was averaged (1) per feather (average among point measurements) and (2) per individual (average among feathers). The repeatability of assessing coloration was very high (97.6% of among-individual variance) as shown by the repeated measurement of coloration of 14 individuals twice one year apart.

Molecular analyses

Genomic DNA from all 373 individuals was extracted from the basal 1 mm of breast feather quills. Extractions were performed either on a BioSprint 96 extraction robot using the BioSprint 96 DNAblood kit or using the DNeasy blood and tissue kit, following the manufacturer's protocols (Qiagen, Hilden, Germany).

To estimate neutral genetic differentiation among barn owl populations, individuals were genotyped at seven polymorphic microsatellite loci (Ta-206, Ta-210, Ta-216, Ta-218, Ta-220, Ta- 306, and Ta-414, Burri et al. 2008). Polymerase chain reactions (PCR) were performed in two PCR multiplexes (Table S1). Multiplex PCR reactions were run in a final volume of 8 µL, containing 2.5 µL of Multiplex PCR Kit buffer (Qiagen), 12 ng of DNA, and the

multiplex primer mixes with forward primers fluorescently labeled. PCR conditions included an initial denaturation step at 95◦C for 15 min, 34 cycles of denaturation at 94◦C for 30 sec, primer annealing at 57◦C for 1 min 30 sec, and primer extension at 72◦C for 1 min. A final step at 60◦C for 30 min was used to complete primer extension. Fragment analysis was run on an ABI 3100 automated sequencer using a Gene Scan™ 500 ROX™ size standard and allele lengths were assigned using GENEMAPPER 4.0 software (Applied Biosystems, Foster City, CA).

To account for sex in color analyses, molecular sex determination for all individuals was performed using the method described in Py et al. (2006). This method allows distinguishing sexes based on a length dimorphism between sex chromosomes in a segment of the *SPINDLIN* gene. In total, 187 males and 186 females were used in the analyses; details per populations are reported in Table 1.

Analyses of population structure

Data from all seven microsatellite markers were used to estimate the levels of differentiation among all populations. After verifying that populations were in Hardy–Weinberg equilibrium (FSTAT 2.9.4, updated from Goudet 1995) and checking for the presence of null-alleles (MICRO-CHECKER 2.2.3, Van Oosterhout et al. 2004), we computed F_{STs} (Weir and Cockerham 1984), to estimate neutral genetic differentiation between populations. Confidence intervals were estimated by running 1000 bootstrap iterations over loci. In addition, we estimated the differentiation statistic D_{EST} between populations (Jost 2008) and Slatkin's R_{ST} (Slatkin 1995). In contrast to F_{ST}, D_{EST} partitions total genetic variance into statistically independent within- and between-population components and

thereby guards against deflated differentiation measures that can arise in measures such as F_{ST} if within- exceeds between-population genetic diversity (Jost 2008). R_{ST} (Slatkin 1995) accounts for microsatellite mutation pattern and is better suited than F_{ST} when mutation is important relative to migration (Slatkin 1995; Balloux and Goudet 2002). To test for a geographic structure of isolation-by-distance, we plotted neutral genetic differentiation (F_{ST}, D_{EST}, and R_{ST}) against the geographic distance between populations. Significance of the regression was tested by a nonparametric, permutation-based, Mantel test, running 1000 bootstrap iterations. Pairwise R_{ST} were estimated in Arlequin 3.1 (Excoffier et al. 2005). All other analyses were conducted in FSTAT 2.9.4 or the R package HIERFSTAT (Goudet 2005).

To test if plumage color differed significantly between populations and sexes, we conducted a two-way analysis of variance (ANOVA). An analysis of covariance (ANCOVA) was then used to test for clinal variation in mean coloration, entering the populations' distance to the southernmost population (Évora, Portugal), sex, and their interaction as independent variables.

Comparing coloration and neutral genetic differentiation

Testing whether a phenotypic cline evolved by neutral processes or by selection requires that phenotypic variation is compared to the neutral patterns of evolution driven by population history and demography. Although migration and genetic drift have an equal effect all over the genome, selection affects only regions harboring the quantitative trait loci (QTL) underlying the phenotypic trait it acts on. Thus if selection causes divergent evolution of phenotypes among populations, either because selection is exerted on coloration itself or on genetically correlated traits, phenotypic differentiation is expected to exceed neutral

differentiation, especially if populations remain interconnected by gene flow such as in cline models. Contrasting the geographic structures of F_{ST} and Q_{ST}, that is, contrasting the respective correlations of F_{ST} and Q_{ST} with geographic distance between pairs of populations constitutes a more robust test of geographically gradually varying selection than comparing overall value of Q_{ST} and F_{ST}. Indeed the latter comparison does not depend on the absolute magnitudes of phenotypic and genetic differentiation. With this approach, selection is inferred from a significant difference between the slopes of the respective regressions: if geographically disruptive selection is stronger than the homogenizing effect of gene flow, phenotypes will diverge more markedly among populations with increasing distance than populations differ at neutral genetic markers.

Q_{ST} calculation requires experimental estimates of additive genetic variances (in common gardens for example), but for many species such estimates are impossible to obtain for logistical reasons. So in practice, many authors calculated $Q_{ST}$with assumptions on the determinism of the trait under study and tested the sensitivity of the results to those assumptions (see e.g., Saether et al. 2007 and references therein). To make the strict distinction between those surrogates of Q_{ST} and "true" Q_{ST}, Saether et al. (2007) proposed to call them P_{ST} (for phenotypic or pseudo-Q_{ST}). Melanic color, such as the pheomelanic trait we analyzed here, is often not dependent on nutritional intake, but on mostly genetically determined melanin deposition (Mundy 2006). Although in the barn owl most of the variation in coloration is genetically determined at least in Switzerland, such as shown by cross-fostering experiments (h^2 = 0.81 ± 0.09, Roulin et al. 1998; Roulin & Dijkstra 2003), we did not experimentally estimate additive genetic variance for barn owl color in this study. We therefore are strict by referring differentiation in plumage coloration to P_{ST} rather than Q_{ST}.

P_{ST} is a function of the within- (σ_w^2) and between-population phenotypic variances (σ_b^2), heritability (h^2), and the proportion of the between-population phenotypic variation due to additive genetic effects (g, and 1 − g corresponds to the environmental effect). Pairwise P_{ST}-values for color were calculated as follows (Wright 1951; Spitze 1993):

$$P_{ST} = \frac{g\sigma_b^2}{g\sigma_b^2 + 2h^2\sigma_w^2},$$

Within- and between-population phenotypic variances were assessed by extracting mean squares (MS) from a two-way ANOVA on color, with factors including population and sex. Within-population MS are an unbiased estimate of the within-population variance (σ_w^2). Between-population variance (σ_b^2) can be estimated as

$$\sigma_b^2 = \frac{MS_b - MS_w}{n_0},$$

where MS_b and MS_w are the within- and between-population MS. n_0 is a weighted average of sample size for each comparison and following Sokal and Rohlf (1995, p. 179–217) is calculated as

$$n_0 = \frac{1}{a-1}\left(\sum^n n_i - \frac{\sum_a n_i^2}{\sum_a n_i}\right),$$

where a is the number of populations to be compared and n_i the number of individuals in the i^{th} population. For further details about the procedure we refer to Storz (2002).

To estimate the effect of geographic distance on color differentiation between populations, we plotted pairwise P_{ST} against pairwise geographic distances between populations. As for neutral genetic differentiation, significance of the regression was tested using the nonparametric, permutation-based Mantel test. Finally, to investigate whether selection was involved in the evolution of the color cline, we tested whether population history alone explained the spatial structure of phenotypic differentiation, or whether the latter persisted if phylogeographic effects inferred from neutral genetic variation were accounted for. Tests of this kind usually involved partial Mantel tests among P_{ST} as a response matrix and F_{ST} and geographic distances as first and second explanatory matrices, respectively (Storz 2002; Saether et al. 2007). However, P_{ST} and F_{ST} are supposed to be identical under the null hypothesis of absence of selection on color or genetically correlated traits. Thus, a simple way to test this hypothesis is to contrast the matrix of pairwise differences between P_{ST} and F_{ST}, $P_{ST} - F_{ST}$, with the matrix of geographic distances. Under our null hypothesis, the two matrices should be uncorrelated, and a positive correlation would indicate a strong signal that selection is acting on the color polymorphism or correlated traits.

Heritability h^2 and the between population additive genetic component g are often different from 1. Following Roulin and Dijkstra (2003), heritability was set to 0.81 and, as an assumption, g to 1 in the first place (Fig. 2, bottom left). However, we tested the robustness of our results with respect to different hypotheses on the proportion g of phenotypic variance. The above analyses thus were repeated by varying g between values of 1 (all

phenotypic variance due to additive genetic effects) and 0.01 (1% phenotypic variance due to additive genetic effects), which is a broader interval than generally tested in comparable studies (Storz 2002, g = 0.15–1; Saether et al. 2007, g = 0.05–1). In this sensitivity analysis, we fixed h^2 to 1 given that Roulin and Dijkstra (2003) found very high heritability in a Swiss barn owl population, and that overestimation of h^2 leads to a deflation of P_{ST}, which is a conservative bias for the question addressed here.

Results

Neutral genetic population structure

The neutral genetic structure of barn owl populations across Europe was very low but significant, with an overall F_{ST} of 0.011 (99% confidence interval 0.007–0.016). Despite the weak population structure, a slight but significant pattern of isolation by- distance was found, indicated by the positive correlation between pairwise F_{ST} and geographic distances between populations (Mantel test: R^2 =0.175; P=0.001) (Fig. 2, top left). Similar differentiation and isolation-by-distance was found for D_{EST} (Fig. 2, top right) and R_{ST} (data not shown), except that sampling variance was high for the latter. Therefore, only F_{ST} was used in the following analyses, as it is statistically directly comparable to P_{ST}. Furthermore, Balloux and Goudet (2002) showed that F_{ST} is better suited than R_{ST} when population structure is low and when microsatellites violate the stepwise mutation model such as is the case for some of the markers used in the present study.

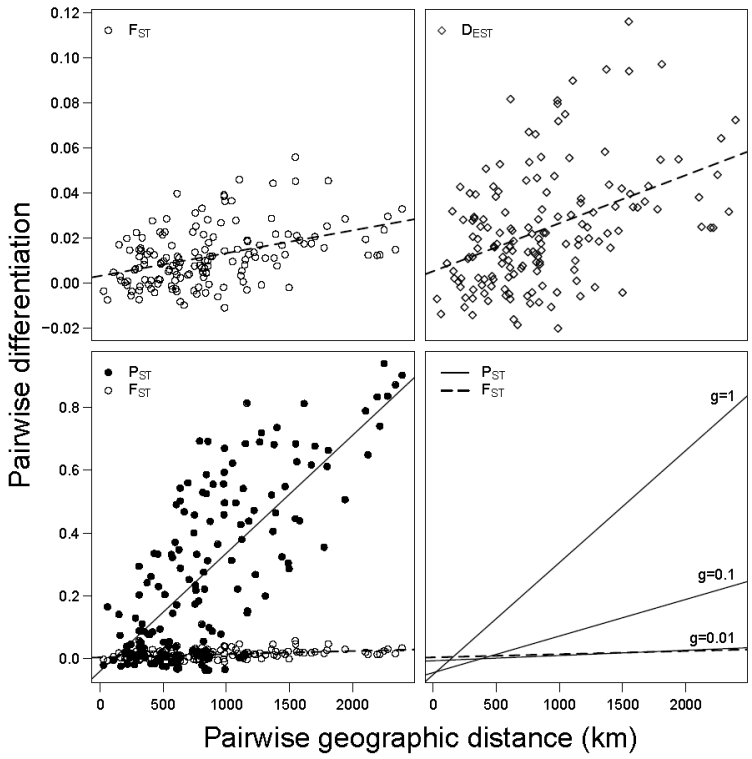

Figure 2. Graphs showing the neutral genetic and color differentiation between populations. Shown are the linear regressions of differentiation against geographic distance between pairs of populations. Top panels: neutral genetic differentiation in terms of F_{ST} (left) and D_{EST} (right). Bottom left panel: color (P_{ST}; h^2= 0.81, g = 1) and neutral genetic differentiation (F_{ST}). Bottom right panel: sensitivity of P_{ST} against the variation of the proportion of the between-population phenotypic variance due to additive genetic effects (g). P_{STs} are indicated by a solid line for three values of g. Points are not drawn for clarity. Phenotypic differentiation increases significantly more with distance than neutral genetic differentiation, even when only 1% of the phenotypic variance observed between populations is due to additive genetic effects (g = 0.01).

Geographic structure of coloration

Our study confirmed previously reported patterns of barn owl color variation quantified by museum skin measurements (Roulin 2003; Roulin et al. 2009). Mean plumage color per population, measured in terms of brown chroma, varied between 0.253 and 0.325 and marked differences of mean coloration were found both among populations and among sexes (two-way ANOVA, population: F = 19.331, P < 0.001, sex: F = 14.078, P = 0.002). A strong clinal pattern of color variation was found when plotting the mean color per population by sex against distance from Évora, Portugal (Fig. 3). An analysis of covariance revealed significant effects of distance from Évora and sex, but no differences in geographic

variation between sexes (ANCOVA, distance from Évora: t = 9.687, P < 0.001; sex: t = −2.607, P = 0.014; interaction distance from Évora∗sex: P = 0.99). Accordingly, the geographic structure of plumage color was very high, with an overall P_{ST} of 0.353 (with h^2 = 0.81, following Roulin and Dijkstra 2003 and g = 1).

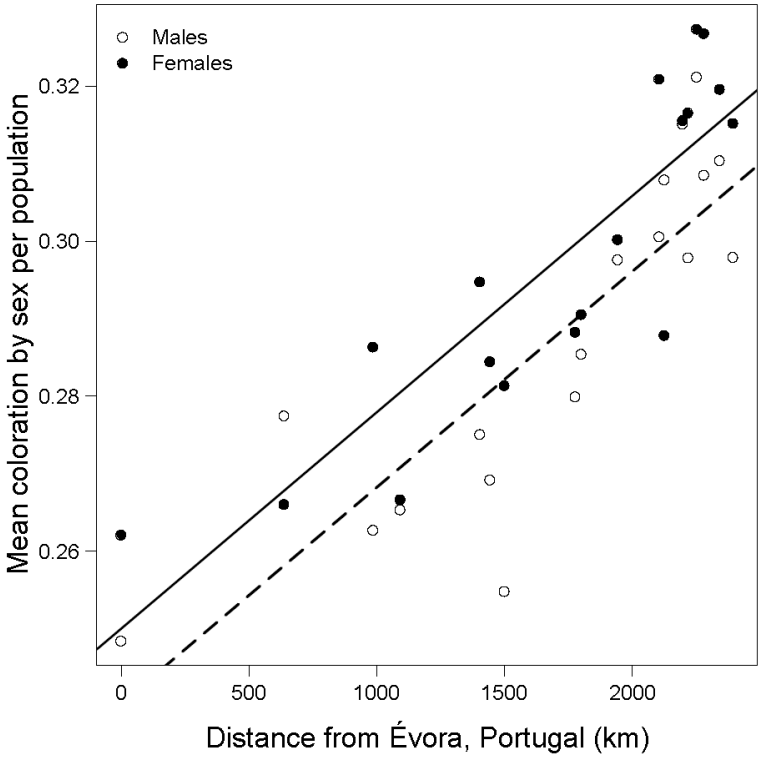

Figure 3. Change of mean coloration across Europe. Shown is the linear regression of mean coloration by sex per population against the distance of each population to the south-westernmost population (Évora, Portugal). Male's values are depicted by open circles and a dashed line, and female's values by filled circles and a solid line.

Comparing coloration and neutral genetic differentiation

The overall differentiation between populations was more than 30-fold larger for color (P_{ST} = 0.353) than for microsatellites (P_{ST} = 0.011). The linear regression of pairwise PSTs against pairwise geographic distances between populations revealed a very strong and highly significant pattern of isolation-by-distance on color differentiation (R^2 = 0.551; P = 0.001) (Fig. 2, bottom left). More importantly, the positive correlation between pairwise

$P_{ST} - F_{ST}$ differences and pairwise geographic distances demonstrates that the isolation-by-distance observed on color differentiation holds when eliminating the baseline level of differentiation resulting from historical and demographic factors (Mantel test R^2 = 0.528; P = 0.001). To test whether these results were robust against changing assumptions on h^2 and g, we tested the robustness of our results to those assumptions in a sensitivity analysis (Fig. 2, bottom right). As aforementioned, a heritability of one is the most conservative assumption to demonstrate that P_{ST} exceeds F_{ST}. By testing values of g between 1 and 0.01, we showed that even when only 1% of the variance between populations is due to additive genetic effect, there is still a significantly stronger isolation-by-distance on color than on the neutral genetic markers (R^2= 0.043; P = 0.013). This analysis thus showed that our conclusion on the involvement of selection in color differences among population is extremely robust to any realistic assumptions on the determinism of the trait.

Discussion

Based on the comparison of the geographic differentiation of coloration to the neutral genetic population differentiation throughout Europe, we show that the barn owl's clinal pheomelanic coloration is not the result of genetic drift, but the most likely result of local adaptation. In accordance with observations from many other birds species (Crochet 2000 and references therein), the neutral genetic structure among European barn owl populations is low (overall P_{ST} = 0.011). The weak but significant pattern of isolation-by-distance suggests that regular effective migration leads to extensive and spatially weakly restricted admixture of neutral genetic variation among populations. This conclusion is supported by barn owl ring recovery data (Cramp 1985; Paradis et al. 1998; Marti 1999). A

recent analysis (following Paradis et al. 1998) of data provided by the Swiss Ornithological Institute (Sempach, Switzerland) revealed a mean dispersal distance of 65 km (median 23 km) with a standard deviation of 121 km (n = 321) and dispersal movements as far as several hundreds of kilometres. Although in theory more detailed insights into the genetic population structure and quantitative measures of gene flow could be obtained from genetic data using clustering approaches or coalescent-based methods, we refrained from performing these analyses, as neither of them is expected to provide satisfying inference of population structure or migration rates with data from almost continuous populations with weak differentiation (F_{ST} = 0.011) and isolation-by-distance as observed in our data (Falush et al. 2007; Faubet et al. 2007).

Given the high rates of gene flow uncovered by genetic analyses, coloration is expected to be homogenized among European barn owl populations if such coloration evolved by purely neutral processes. However, color differentiation remains strong after accounting for neutral genetic population differentiation, suggesting that strong recurrent selection on coloration or genetically correlated traits is involved in the maintenance of the clinal coloration polymorphism. This conclusion remains consistent even when coloration is assumed to be completely heritable (h^2 = 1) and to its largest extent dependent on variation in environment between populations (g = 1%, i.e., 99% of the variation between population is due to environmental effects). Experimental evidence from Swiss barn owls shows very limited environmental dependence of melanin-based coloration (Roulin and Dijkstra 2003). The parameter space used in our sensitivity analysis is thus far beyond realistic estimates of the environmental component (1 – g) and conservative for heritability (h^2). Pujol et al. (2008) recently criticized the use of in situ phenotypic measures from natural populations to infer selection (the P_{ST} approach). The conducted sensitivity analyses permit to settle this

problem, and the congruence of the results obtained by F_{ST} and D_{EST} confirms the weak population structure indicated by genetic data not being an artifact of high within-population genetic diversity.

As demonstrated, our data rule out that the color cline established as a result of spatially restricted gene flow. However, patterns closely resembling the ones expected from selection acting along an environmental gradient can be generated by the neutral process of surfing that has found little attention in the study of phenotypic clines so far (Klopfstein et al. 2006; Excoffier and Ray 2008). Alleles responsible for whitish or reddish-brown colorations could have gradually increased in frequency by genetic drift acting at the front of a past spatial population expansion out of either end of today's cline. Although surfing might be a frequent phenomenon leading to phenotypic clines, especially when starting from standing genetic variation (Excoffier and Ray 2008), several findings argue against this neutral scenario in the case of the barn owl color cline. (1) Experimental evidence suggests that coloration indeed is of functional relevance (Roulin 2004; Roulin and Altwegg 2007; Roulin et al. 2008). (2) Long-range dispersal as observed in the barn owl renders surfing unlikely compared to species that disperse over short distances in a stepping-stone-like manner. (3) Apart from clinal variation on the European continent, color clines in the barn owl independently evolved in North and South America and in Africa (Roulin et al. 2009). As surfing is a stochastic process affecting random regions in the genome, it appears unlikely that coloration would have been involved in surfing events four times independently. (4) Last and most importantly, surfing would be most likely if the colonization of Europe after the last glaciation occurred out of a single refugium. However, as already suggested by Voous (1950) half a century ago and confirmed by preliminary mitochondrial data obtained in our laboratory (S. Antoniazza, R. Burri, L. Fumagalli, J. Goudet, and A. Roulin, unpubl.

data), Europe seems to have been colonized from at least two regions. Even though postglacial colonization might have brought into secondary contact two distinct color morphs that evolved in allopatry, and the color cline in first place could have established neutrally by admixture, the maintenance of the cline despite extensive gene flow requires the recurrent action of selection.

Spatially heterogeneous selection and cline evolution

The most eminent question for the evolution of phenotypic diversity in species with continuous repartition across whole continents is how selection can restrict homogenization of phenotypic traits in the presence of high rates of gene flow (Nosil 2008; Räsänen and Hendry 2008). In barn owls, several lines of evidence suggest that both indirect selection and direct selection are involved in the maintenance of the color polymorphism. Reddish-brown individuals invest more into parental care (Roulin et al. 2001; Roulin 2006) and they grow faster in body mass than white ones when rearing conditions are relaxed (Roulin et al. 2008). The linkage of color to life-history components in this case seems to result from physical linkage or pleiotropy of the respective genes (see also Roulin 2006; Ducrest et al. 2008), whereas the correlation of the color polymorphism with diet (Roulin 2004) may establish by direct selection on color as a consequence of different foraging success upon alternative prey. Altogether, this indicates that barn owl color phenotypes occupy different ecological niches (Roulin 2004; Roulin and Altwegg 2007; Roulin et al. 2008), suggesting that divergent selection among niches maintains the color cline observed at the continental scale.

Depending on the niche distribution across the continent, different scenarios may explain the evolution of the color cline. In the simplest model, selection might be exerted by

an environmental gradient creating locally homogeneous niches. In each region individuals of nonadapted phenotype are counter-selected, but strong recurrent immigration maintains phenotypic variation. Close inspection of the patterns of color variation in barn owls reveals that even though mean coloration tightly fits a linear geographic cline, color variation within populations is usually extensive. It seems thus more likely that habitat is also heterogeneous at the local scale, with most niches present all over the continent, but at gradually changing frequencies. Immigrants of any phenotype may thus settle and reproduce almost throughout the continent. The maintenance of the color cline in such heterogeneous landscapes involves different processes: (1) ecological selection prevents invasion of niches by nonadapted phenotypes, and (2) niche frequency and competition for niches determine the local frequencies of phenotypes, and thereby the local mean coloration. The strong phenotypic differentiation at the continental scale would be the result of local ecological selection acting in conjunction with a cline in niche frequency and selective pressure. Populations may then even almost freely exchange neutral genetic diversity, because selection intensity on progeny of subsequent generations will rapidly decrease with increasing level of back-crossing when mating is most likely with locally adapted individuals. Additional sampling effort, spatially explicit simulations of selection and population-level radio-telemetry observations in conjunction with the use of high-resolution environmental maps will help elucidate the spatial scale at which the selection pressures involved in barn owl color evolution are acting.

Local adaptation at large spatial scales

The present study represents a striking illustration of the levels of phenotypic differentiation that can be achieved in nature despite substantial gene flow. Although the patterns observed in the barn owl might seem particular, we believe that they are far from being restricted to this species. Rather, we expect them to be ubiquitous in nature, especially in species with large distributions and high dispersal propensity. However, so far only a handful of studies combined measures of phenotypic and neutral genetic variation in natural populations displaying phenotypic clines at continental scales (examples include Long & Singh 1995; Merilä 1997; Gockel et al. 2001; Storz 2002; Palo et al. 2003; Ingvarsson et al. 2006; Savolainen et al. 2007; Demont et al. 2008). Compared to the linear gradients and large geographic distances in these cases (>1000 km), other studies that identified phenotypic clines rather reported sharp transition zones of limited width relative to the species' distribution, with phenotypes changing quickly across hybrid zones (reviewed in Barton and Hewitt 1985) or ecotones (e.g., Mullen & Hoekstra 2008). We put forward that the extent and pronounced linearity of the phenotypic clines and phenotypic isolation-by-distance despite almost absent neutral genetic population differentiation observed in the former studies and in the barn owl are likely a matter of spatial scale. At large spatial scales, the environment varies in numerous ecological dimensions that constitute likely selective agents, and local adaptation at these scales may seem inevitable. Working at such scale permits to minimize the influence of local variation in environmental conditions relative to their variation over the entire study area. As moreover many important ecological parameters are expected to vary linearly across continents, clear-cut patterns of isolation-by-distance on phenotypic traits may easily emerge at large spatial scales, because distance is a good proxy for environmental variation. The confirmation of the action of selection

acting at large spatial scales may thus be straight forward. However, the identification of the selective agents such as climatic and ecological variables, in turn, is tremendously flawed with problems of spatial autocorrelation. Finally, a combination of approaches integrating various spatial scales and methods such as landscape genetics will be essential to get track of the detailed selective agents involved in the process of local adaptation.

Conclusion

We believe that the barn owl is a well-suited model for the study of the interplay of gene flow and selection, which in turn is of central importance to an increased understanding of the processes of local adaptation and speciation. This species represents one of only six worldwide distributed bird species, and there may be only few more vertebrate species with comparable autochthonous areas. This implies that the species encounters various environmental conditions, and we expect local adaptation to be common. Might the species show a special propensity for adaptation that explains its global success? The identification of selection, such as conducted here for color, provides only the first essential step toward the understanding of how local adaptation evolves. The world-wide distribution provides exceptional opportunities to confirm hypotheses derived from single populations and compare patterns and processes of phenotypic and genomic evolution among allopatric populations. In-depth studies of phenotype frequencies within populations and detailed description of the color cline in conjunction with the establishment of the species' phylogeography will provide important information on the evolutionary history of the color cline in Europe and the spatial scale at which selection is acting. Finally, tracking down color evolution to the molecular level will allow us to study whether in

allopatric populations the same underlying genes and processes are involved in barn owl color evolution.

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Supplementary material

Table S1. Primer concentrations for multiplex PCR reactions.

	Locus	Primer concentration [mM]
Multiplex 1	Ta206	0.220
	Ta210	0.052
	Ta216	0.067
	Ta306	0.082
Multiplex 2	Ta-218	0.089
	Ta-220	0.055
	Ta-414	0.136

Chapter 2: Natural selection in a post-glacial range expansion: the case of the colour cline in the European barn owl

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Abstract

Gradients of variation – or clines – have always intrigued biologists. Classically, they have been interpreted as the outcomes of antagonistic interactions between selection and gene flow. Alternatively, clines may also establish neutrally with isolation-by-distance or secondary contact between previously isolated populations. The relative importance of natural selection and these two neutral processes in the establishment of clinal variation can be tested by comparing genetic differentiation at neutral genetic markers and at the studied trait. A third neutral process, surfing of a newly arisen mutation during the colonisation of a new habitat, is more difficult to test. Here, we designed a spatially-explicit ABC simulation framework to evaluate whether the strong cline in the genetically-based reddish coloration observed in the European barn owl (*Tyto alba*) arose as a by-product of a range expansion or whether selection has to be invoked to explain this colour cline, for which we have previously ruled out the actions of isolation-by-distance or secondary contact. Using ABC simulations and genetic data on 390 individuals from 20 locations genotyped at 22 microsatellites loci, we first determined how barn owls colonized Europe after the last glaciation. Using these results in new simulations on the evolution of the colour phenotype, and assuming various genetic architectures for the colour trait, we demonstrate that the observed colour cline cannot be due to the surfing of a neutral mutation. Taking advantage of spatially explicit ABC, which proved to be a powerful method to disentangle the respective roles of selection and drift in range expansions, we conclude that the formation of the colour cline observed in the barn owl must be due to natural selection.

Introduction

Determining the relative roles of natural selection and neutral processes as driving agents of evolutionary change has long been the focus of discussions in the field of evolutionary biology (Kimura 1983; Nei 2005; Wagner 2008). A process of particular interest in this context is one observed in many species presently occupying temperate areas: range expansions. Most (if not all) species currently inhabiting Europe and North America have undergone postglacial recolonisation events, increasing their ranges and population sizes (Hewitt 2000), and nowadays, some extant species and populations facing the on-going climatic changes and human alterations to the environment may also respond by increasing their range (Parmesan & Yohe 2003). Range expansions are a key factor for the discussion above because they often take place over an environmental gradient, which potentially provides natural selection with the opportunity to generate locally-adapted variants (Hewitt 1996). When these variants are distributed gradually across the environment, a cline is formed (Endler 1977). Clines along the path of range expansions, however, can also be formed without natural selection. The series of founder events, which are inherent to the colonisation of new areas (Currat & Excoffier 2005), may lead to the formation of allele frequency clines simply through the neutral process of allele surfing (Edmonds et al. 2004; Klopfstein et al. 2006). Even though other neutral processes could also lead to the formation of clines in range expansions [e.g. demic diffusion (Cavalli-Sforza et al. 1993), kin-structured migration events (Fix 1997)], allele surfing is arguably the most likely to take place in the case of the barn owl. In this process, neutral alleles may “surf” the wave of range expansion, increase their frequency along the way eventually forming a genetic cline. Allele surfing can happen with standing genetic variants (Klopfstein et al. 2006) or, as it was first described, new mutations. If the underlying genetics has any effect on phenotype, a purely neutral cline

may become very similar to what one would expect to be a selection-derived cline (Currat et al. 2006).

Classically, clines have been studied in the context of hybrid zones, a secondary-contact zone between species or populations that evolved in allopatry, where selection against hybrids prevents gene flow and generates clines of phenotypes or alleles frequencies (Barton & Hewitt 1985). This is well described in the hybrid-zone literature, where the terms "cline" and "hybrid zone" are even sometimes confounded (Barton & Hewitt 1985). The processes behind the formation of such clines have been investigated in some details both theoretically and experimentally (Barton & Gale 1990; Barton & Hewitt 1985; Gay et al. 2008). Clines could also be the result of the mixing of populations adapted to different ecological conditions where the ecological transition occurs over short distances [e.g. latitudinal clines (James et al. 1997) or sharp environmental changes (Mullen & Hoekstra 2008)]. These ecological clines can be analysed in a similar way to the hybrid-zone clines (e.g. Mullen & Hoekstra 2008). For these types of clines, the development of tools to infer selection has a long history and the method relies on the comparison between the clines' width (w) and species' dispersal distance (σ). In this case, selection is proportional to the square root of σ/w (Linnen & Hoekstra 2009; Slatkin 1973).

Clines can also appear through two neutral processes; isolation by distance and secondary contact without selective disadvantage of hybrids (Novembre & Di Rienzo 2009). When compared with natural selection, these neutral processes can essentially be ruled out by comparing the genetic/phenotypic variation putatively under selection to the neutral genetic variation. If the trait putatively under selection presents a stronger signal of population differentiation [higher Q_{ST}, sometimes referred to as P_{ST} (Antoniazza et al. 2010; Saether et al. 2007)] than neutral genetic markers (F_{ST}), there is probably selection involved

in maintaining or leading to locally-adapted forms (Leinonen et al. 2008; Spitze 1993). Otherwise – if Q_{ST} is not significantly higher than F_{ST} – isolation by distance or secondary contact are enough to explain the observed patterns. Several studies have been performed to either compare differentiation at quantitative traits and neutral markers (Q_{ST}-F_{ST}) (Antoniazza et al. 2010; Demont et al. 2008; Gockel et al. 2001; Hangartner et al. 2012; Long & Singh 1995; Merilä 1997; Palo et al. 2003; Savolainen et al. 2007; Storz 2002), or to compare genetic variation at different types of loci (F_{ST}-F_{ST}) (Ingvarsson et al. 2006; Kooyers & Olsen 2012; Saccheri et al. 2008).

In large-scale clines (occurring over wide geographical ranges, such as a continent), the role of selection has only been tackled through theoretical investigations focusing on either gene frequencies (Bazykin 1969; Endler 1977; Fisher 1950; Haldane 1948), or quantitative phenotypic traits (Barton 1999; Case & Taper 2000; Kirkpatrick & Barton 1997; Leimar et al. 2008; Slatkin 1978). No methods have yet been developed to infer selection in this case. In addition, the empirical studies describing large-scale clines have consistently neglected the evaluation of the surfing phenomenon as their possible cause. They have largely assumed natural selection to be driving force leading to the observed patterns [see Currat et al. (2006) and Vasemägi (2006) for critical reviews, and Kujala (2012) for an exception]. Nevertheless, the most probable source of allele surfing – i.e. range expansions – is common. Most species inhabiting temperate latitudes of both hemispheres spent the last glacial maximum (LGM) in refugia, which were closer to the equator than their current distribution and then expanded their range after the last ice age (Hewitt 1999, 2000; Taberlet et al. 1998).

Evaluating how likely it is for a given cline to originate by allele surfing (relative to natural selection) is essential to understand its biological basis and can bring key insight on

the more general discussion about the prevalence of selective processes in biological evolution. The establishment of clines by allele surfing in range expansions, however, is more difficult to rule out by means of Q_{ST}-F_{ST} comparisons than the other two neutral processes. Surfing mutations may also occur in the loci underlying the candidate trait (Klopfstein et al. 2006), leading to an inflated Q_{ST} when compared to other random loci's F_{ST}. In order to deal with this situation, one possible approach is to first infer/reconstruct the most likely demographic history for the taxon under investigation. This can be done using approximate Bayesian computation [ABC (Beaumont et al. 2002; Bertorelle et al. 2010; Csillery et al. 2010; Sunnaker et al. 2013)], where simulations with variable scenarios and demographic-parameter values are used to infer which parameters are closest to the observed genetic data, and whether the species has undergone a range expansion (Eriksson et al. 2012; Estoup et al. 2004; Estoup & Clegg 2003; Itan et al. 2009; Neuenschwander et al. 2008b; Warmuth et al. 2012). Second, using these estimated demographic parameters, a new round of neutral simulations is carried out, focusing this time on the phenotypic trait showing clinal variation. Taking advantage of many replicates, this procedure allows assessing the probability of the cline under investigation to have been generated by purely neutral processes (i.e. allele surfing in a range-expansion scenario). This idea of using a background demographic model to infer selection process has a long history in population genetics, for example in outlier loci detection approach (Beaumont & Balding 2004; Foll & Gaggiotti 2006), but rely in general on very simple demographic models (island models for example). Examples where a sound investigation and reconstruction of a demographic model has been carried out before inferring selection is less common. But examples can be found for several model species: *Arabidopsis* where such an approach was successfully used by Roux et al. (2012) to infer balancing selection, humans where Tarazona-Santos et al.

(2013) used a demographic model by Laval et al. (2010) to study selection on metabolic genes and *Drosophila* where Singh et al. (2013) measured adaptation rate in a X-linked genomic region. Finally the golden aim is to estimate both selection and demography together, but this goal is still difficult to reach for complex and realistic models (Li et al. 2012), but has for example been successfully conduct by Itan et al. (2009) to reconstruct demographic and selection history on a lactase allele in humans.

One striking example of clinal variation is provided by the south-west/north-east cline in colour of the European barn owl (*Tyto alba*) described by Roulin and colleagues (Roulin 2003; Roulin et al. 2009), and analysed along with neutral genetic markers in Antoniazza et al. (2010). Based on a comparison of the spatial variation of the colour with the neutral genetic diversity, the latter study revealed that the south-west/north-east colour cline is significantly steeper than population differentiation at neutral genetic markers measured in the same populations. Antoniazza et al. (2010) discussed the surfing hypothesis, but did not test it. A major characteristic of neutral genetic diversity in European barn owls is a decline from south-western (Iberian Peninsula) to north-eastern Europe (North-Eastern Germany to Serbia, Fig. S1). The likely origin of this genetic diversity decline is a series of bottleneck events during the post-glacial colonisation of northern Europe. Here, we investigate whether a post-glacial colonisation model is compatible with today's observed genetic diversity of the European barn owl, and investigate how likely it is for the colour cline to have arisen by allele surfing (as opposed to natural selection) during colonisation.

To reconstruct past and current demography of the European barn owl, a dataset of 390 individuals genotyped at 22 microsatellites coming from 20 sampling locations (Fig. 1) in Western Europe was analysed with spatially explicit simulations within an approximate Bayesian computation (ABC) framework. The observed patterns were compared to those

generated with spatially explicit computer simulations using several plausible historical scenarios (Table 1) and 6-9 demographic parameters (Table 2). Based on observed genetic patterns, classical phylogeographic analyses and ecological knowledge of the species, a scenario consisting of a single colonisation from the Iberian Peninsula was hypothesized. As geographic variation in genetic diversity might arise by other processes than colonisation, we also tested scenarios with a south-west/north-east gradient of effective population size and extinction rate. Additionally, considering that many species were shown to have more than one glacial refugium (Taberlet et al. 1998), we looked at models with two glacial refugia in the Iberian Peninsula and in Greece. Finally, to control for the possibility that the patterns observed might not be derived from a colonisation process, several models without colonisation were tested as well.

Using the parameters obtained for the best-supported scenario for neutral genetic markers, we ran additional simulations to model the evolution of the colour trait. Different possible genetic architectures underlying the colour trait were investigated. For each one of these, we estimated the probability of generating a cline as steep as the one observed in the natural populations without selection.

Table 1. Demographic models tested with ABC for the demographic history of the European barn owl. Two dimensions of the models are described (colonisation and heterogeneity) with the each model's number of variable parameters.

Colonisation model	Heterogeneity model	Nb. of varying parameters (i.e. with prior distributions)
One-refugium **(Iberian)**	*One-carrying-capacity* (base model)	6
	Carrying-capacity-cline (SW-NE)	7
	Extinction-rate-cline (SW-NE)	8
	Two-migration-rate (one during colonisation and one at carrying capacity)	7
Two-refugium **(Iberian and Greek)**	*One-carrying-capacity*	7
	Carrying-capacity-cline (SW-NE)	8
	Extinction-rate-cline (SW-NE)	9
	Two-migration-rate (one during colonisation and one at carrying capacity)	8
No-colonisation	*One-carrying-capacity*	6
	Carrying-capacity-cline (SW-NE)	7
	Extinction-rate-cline (SW-NE)	8

Material & Methods

I. Sampling and molecular analyses

From 20 locations throughout Europe, a total of 390 barn owls were sampled by collaborators working in survey programs, recovery centres and museums (Fig. 1). Genomic DNA was extracted from the basal 1 mm of breast feather quills, or from blood or muscles stored in 96% ethanol. Extractions were performed either on a BioSprint 96 extraction robot using the BioSprint 96 DNA blood kit or using the DNeasy blood and tissue kit, following the manufacturer's protocols (Qiagen, Hilden, Germany).

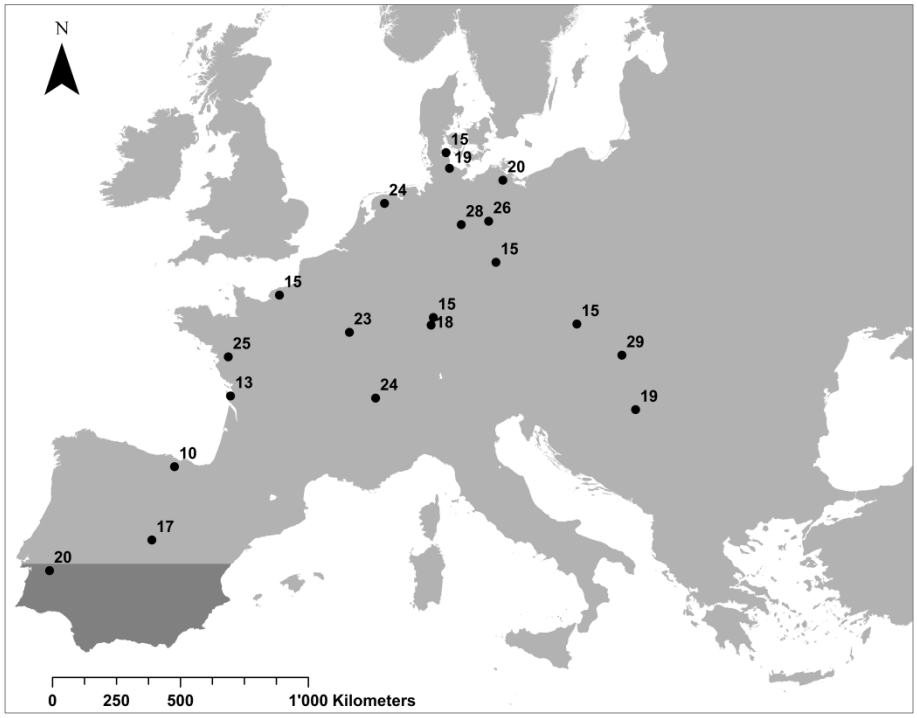

Figure 1. Map of the sampling locations and sampling sizes. Sampling sizes and sampling locations for the observed dataset are indicated. Similar sampling locations and sampling sizes are generated for the simulated dataset. The Iberian glacial refugium demes are indicated in dark grey. We use a Europe Albers Equal Area Conic projection to adequately represent surfaces (Snyder 1987).

Population genetic statistics were estimated from genotypes obtained for 22 polymorphic microsatellite loci [(Ta-202, Ta-204, Ta-206, Ta-210, Ta-212, Ta-214, Ta-215, Ta-216, Ta-218, Ta-220, Ta-305, Ta-306, Ta-310, Ta-402, Ta-408 and Ta-413 from Burri et al. 2008) and (54f2, Calex-05, FEPO42, Oe053, GgaRBG18 and Tgu06 from Klein et al. 2009)]. Polymerase chain reactions (PCR) were performed in five multiplexes using the QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany) and the following protocol: initial step of denaturation for 15 min at 95 °C, 34 cycles of 30 sec denaturation at 94 °C, annealing for 1.5 min at 57 °C, and elongation at 72 °C for 1 min. Final elongation for 30 min was conducted at 60 °C. The primer concentration and multiplexes composition can be found in Table S1. Fragment analyses were run on an ABI 3100 sequencer with a ROX 500 size standard and allele lengths were assigned using GENEMAPPER 4.0 (Applied Biosystems, Foster City, CA, USA). After verifying that no null-alleles were present (MICRO-CHECKER 2.2.3, Van

Oosterhout et al. 2004) and that populations were not showing departure from Hardy-Weinberg equilibrium (Goudet 1995) the dataset was used to calculate observed summary statistics for the ABC estimation procedure. All summary statistics for both observed and simulated data were calculated using quantiNEMO (Neuenschwander et al. 2008a) and custom R (R Development Core Team 2008) scripts.

II. Approximate Bayesian computation (ABC)

1. Population genetics patterns and choice of summary statistics

The rationale behind ABC is to compare simulated genetic data obtained under various scenarios and demographic/genetic parameters against observed genetic data through summary statistics (Beaumont 2010; Beaumont et al. 2002; Bertorelle et al. 2010; Csillery et al. 2010; Sunnaker et al. 2013). The choice of summary statistics on which the comparison is based is thus a key component of an ABC analysis. The summary statistics should describe the genetic data sufficiently, but should also be kept to a minimal number: Each additional summary statistic adds both information and noise to the parameter estimation (Beaumont et al. 2002).

The present data exhibit strong geographic patterns of genetic diversity and population structure, which can be summarized by few summary statistics: (i) a significant signal of isolation-by-distance [IBD; pairwise F_{ST} as a function of pairwise geographic distances, Fig. S2 (Mantel test, $R^2 = 0.310$, $p < 0.001$)], and (ii) a significant reduction in genetic diversity from south-west to north-east [mean allelic richness per population as a function of geographic distance from the south-western most population, Fig. S1 ($R^2 = 0.779$, $p < 0.001$)]. Four statistics were implemented to summarize these patterns: (i) The IBD slope

(5.68×10^{-4}); (ii) average mean pairwise F_{ST} between populations (1.68×10^{-2}); (iii) the slope of the regression of the mean allelic richness per population as a function of its distance to the south-western-most population (-2.18×10^{-2}); and (iv) the average mean allelic richness per population (5.32).

2. Base model

One of the major drivers of barn owl populations' dynamics is winter harshness (Altwegg et al. 2006; Marti & Wagner 1985; Massemin & Handrich 1997). The sensitivity of this species to climate, notably to long periods of snow cover, is well known. There is no doubt that European barn owls endured the LGM in refugia in ice- and largely snow-free ranges south of their current European distribution. The strong cline in genetic diversity from south-west to north-east Europe points toward a single colonisation from the Iberian Peninsula (or north-Africa via Gibraltar, Fig. S1). Our basal simulation model is thus based on a colonisation of Europe from a single, Iberian glacial refugium.

Simulating colonisation processes requires spatially explicit modelling. A modified version of the quantiNEMO programme was used to simulate the colonisation and the resulting neutral genetics (Neuenschwander et al. 2008a), using an integrated coalescent layer for increased efficiency. Our simulations consisted of two phases, similar to the approach implemented in SPLATCHE (Currat et al. 2004; Ray et al. 2010), but with extra features (e.g. possibility to simulate quantitative traits). In a first phase, spatially explicit demographic history was simulated forward in time (starting with the post-glacial colonisation and ending today). In this phase the demographic history of populations is simulated based on the demographic parameters presented in Table 2. In the second phase genetic data were generated in a coalescent approach (backward in time, starting from

today's sample and going back to the most recent common ancestor of all sampled lineages) using the demographic information obtained from the demographic simulations (Hudson 1990; Nordborg 2001). The genetic data (22 unlinked microsatellite markers) was simulated for the same number of individuals and populations as in the observed data. Mutations followed a stepwise mutation model (SMM).

Simulations were performed on a raster map of Europe consisting of 2671 square land demes, each 50 km × 50 km in size (Fig. S3). The deme size was chosen to correspond to the dispersal abilities of the species. In the Netherlands, about 30% of the juveniles disperse more than 50km (Bunn et al. 1982) and the deme size chosen permits to be at a scale for which dispersal values can be well estimated (see below details on the definition of the prior for migration).

Simulations started with a single population in the glacial refugium in the south of the Iberian Peninsula. At the start of a simulation, this refugium population was distributed in equal numbers among the nearest 100 demes (Fig. S3). In the following generations, the population range expanded successively across Europe based on demographic processes, such as local logistic population growth and migration to the four neighbouring demes (stepping-stone migration model). This described base model requires five demographic parameters (time of the onset of colonisation, migration rate, deme carrying capacity, size of the refugium population, and intrinsic population growth rate) and a single genetic parameter (mutation rate of the microsatellites, Table 2).

3. Prior distributions

Even if plenty of information is available on the barn owl biology in general, we chose to use uninformative priors in order to extract the information present directly in our

population genetic dataset. For most priors, uniform distributions were chosen and their limits where defined with the available ecological data on the species. For two parameters, carrying capacity and mutation rate, a lognormal distribution seemed more appropriate for concentrating the parameter search on the lower values of these parameters. Also, the prior-distribution densities where defined using available ecological literature (see details bellow).

- Start of the colonisation (time): As a result of high sensitivity of barn owls to winter harshness, the colonisation of the northern part of Europe necessarily occurred after the warming of the continent, i.e. after the LGM around 20 000 years ago (Clark et al. 2009). Since no information on the onset of colonisation is available, a broad uniform prior was chosen ranging from 2000 to 10 000 generations, which is about 7200-36 000 years BP assuming a constant generation time of 3.6 years for barn owls (Altwegg et al. 2006).
- Migration rate: Migration rate is generally high in the barn owl. In the Netherlands, more than 30% of the juveniles disperse more than 50 km from their place of birth to their place of reproduction (Bairlein 1985; Bunn et al. 1982). We account for these dispersal distances by defining a deme size of 50 km × 50 km (see above) and by defining a wide uniform migration prior allowing for high migration rates from 0 to 0.5, where the migration rate represents the proportion of the population in a given deme that emigrate to the four directly neighbouring demes at each generation.
- Carrying capacity: The barn owl census population size is well estimated in Europe and it counts about 140 000 breeding pairs (Hagemeijer & Blair 1997). We chose to cover a broad interval of 5-10 000 individuals per deme (so between 13 355 and 26

710 000 overall), but we put more weight on small values by using a lognormal distribution with a mean of 300 and a variance of 400.

- Size of the refugium population: As no information is available about this population size we used a wide uniform distribution between 100 and 100 000 individuals.
- Population growth rate: We chose a wide uniform prior between 0 and 2. Note, that the growth rate has only an effect during colonisation when population size has not reached carrying capacity. Its effect on the model is therefore limited to this stage of the simulations. The population growth was modelled with a logistic regulation model, where growth rate represent the slope of logistic regression.
- Mutation rate: a lognormal distribution between 10^{-8} and 10^{-2} with a mean of 10^{-3} and a variance of 8×10^{-2} was used as prior to span the full range of plausible mutation rate values (Ellegren 2000).

Table 2. Demographic parameters for the different scenarios (models). Details on the *a priori* value distributions of the different models. Uniform distributions have equal probability of sampling any value between the defined boundaries; lognormal distributions have a higher probability of sampling values closer to its mean in a logarithmic scale, with predefined upper and lower limits (truncated). The brackets describing lognormal distributions give: (lower bound, upper bound, mean, variance).

Parameters	For which model	Prior characteristics
Start of the colonisation	All models	Uniform (2000-10 000 generations)
Population growth rate	All models	Uniform (0-2)
Mutation rate	All models	Lognormal ($1e^{-8}$-$1e^{-2}$, $1e^{-3}$, $8e^{-2}$)
Size of refugium population	All models	Uniform (100-100 000) but for 2-refugia models Uniform (200-100 000) for 1-refugium models
Migration rate	All models but *two-migration-rate*	Uniform (0-0.5)
Migration rate high density	*Two-migration-rate*	Uniform (0-0.5)
Migration rate low density	*Two-migration-rate*	Uniform (0-0.5)
Carrying capacity	All models but *carrying-capacity-cline*	Lognormal (5-10 000, 300, 400)
Carrying capacity of the SW deme	*Carrying-capacity-cline*	Lognormal (5-10 000, 300, 400)
Carrying capacity of the NE deme	*Carrying-capacity-cline*	Lognormal (5-10 000, 300, 400)
Extinction rate SW deme	*Extinction-rate-cline*	Uniform (0-0.5)
Extinction rate NE deme	*Extinction-rate-cline*	Uniform (0-0.5)
Divergence time	*Two-refugium*	Uniform (0-120 000)

4. Model comparison

ABC does not only allow estimating model parameters, but it is also effective in contrasting different models (eg. Sunnaker et al. 2013 and references therein). We took advantage of this feature to test for different scenarios that could explain the barn owl's post-glacial evolutionary history, and then applied the parameter estimation to the best supported model, using the same prior distributions as in the model comparison, but increasing the number of simulations used to 1 million.

Three colonisation models: Our observation of a strong decrease of allelic richness from the Iberian Peninsula towards north-eastern populations (Fig. S1) suggests a single colonisation from the Iberian Peninsula. Our base model (one-refugium model) thus consists of a single colonisation of Western Europe from this Peninsula. However, many taxa in Europe are known to have survived the cold period also in eastern glacial refugia (Hewitt 1999; Taberlet et al. 1998). We tested this hypothesis by adding a second eastern glacial refugium, of identical size situated in Greece (two-refugium model). Finally, we tested the hypothesis of whether barn owls resisted the cold period and remained across Europe and thus had no colonisation phase after the LGM. We implemented this model by directly spreading the initial population size over the whole continent (no-colonisation model).

Four heterogeneity models: The described base model has constant environmental characteristics (one-carrying-capacity model), i.e. deme characteristics did not change over space. However, several ecological aspects of the barn owl, apart from the colonisation, might have induced spatial variation in genetic diversity. Half of the extant European barn owls are breeding in the Iberian Peninsula, and there is a strong decrease in population sizes from south-western to north-eastern Europe (Hagemeijer & Blair 1997). We thus tested whether a model with clinal variation in carrying capacity from south-west to north-east Europe fits the data better (carrying-capacity-cline model). A second key characteristic that might influence the spatial variation in genetic diversity is the variation in the extinction rate. The European barn owl is very sensitive to cold, snow-rich winters, and the gradient of continentality from south-western to north-eastern Europe might play an important role in creating the observed pattern of genetic variation. We thus also ran a model that includes a south-west/north-east cline in extinction rates (extinction-rate-cline model). Both extinction-rate and carrying-capacity clines were implemented by defining independent values for

south-western and north-eastern extremes (sampled from the same prior distributions), with a linear interpolation for the demes along the clines. Finally, the last model investigated is based on the observation that migration rates may differ depending on the stage of colonisation looked at: Migration is often higher during the colonisation and then goes down once carrying capacity has been reached (Neuenschwander et al. 2008b; Saether et al. 1999). In this model (two-migration-rate model), we allowed for two migration rates, one at low density during colonisation and one at high density when demes are completely populated.

The four heterogeneity models were combined with the three colonisation models. The combination of the no-colonisation and two-migration-rate models was not used since the migration rate during colonisation is not part of this model. Eleven different models were therefore compared (Table 1).

For the model comparison in ABC, we run 10^5 simulations for each of the eleven models based on parameters drawn from the corresponding prior distributions (Table 2). Each simulation was compared to the observation by their summary statistics, resulting in a Euclidean distance. Models were then compared based on their posterior probabilities following Leuenberger and Wegmann (2010), as implemented in ABCTOOLBOX (Wegmann et al. 2010).

5. Parameter estimates

The best demographic model was then selected for final parameter estimation. A total of 10^6 simulations were generated as before based on parameters drawn from the prior distributions. The 1000 simulations closest to the observation were retained for

parameter estimation using a locally-weighted linear-regression approach implemented in the package ABCTOOLBOX (Wegmann et al. 2010).

6. Quality assessment of estimates

To test the accuracy of our estimates, we use 1000 randomly chosen simulations (from the 10_6 simulations dataset) with known parameter values and their resulting genetic data as pseudo-observations. Using the same ABC framework as before, we estimated the parameter values for these pseudo-observations. The accuracy of the estimation was measured by comparing the estimated parameter value (mode) against the "true" parameter value using the following statistics: relative root mean square error (RRMSE), mean relative bias, proportion of high posterior density 50% (HPD50%) encompassing the pseudo observed value, proportion of HPD95% encompassing the pseudo-observed value. We also computed the R^2 of the linear regression of the estimated parameter values as a function of the pseudo-observed parameter values (Neuenschwander et al. 2008b).

III. Simulations applied to the colour trait

Additional simulations were performed to assess the probability of neutral processes generating the colour cline observed in the barn owl across Europe. These simulations were run using the best-supported demographic model and parameter values drawn from the posterior distributions therein (HPD95% intervals). In order to simulate colour as a quantitative trait, we ran the simulations forward in time in quantiNEMO (Neuenschwander et al. 2008). For the observed data, colour measurements (phenotypes) were obtained for each individual by sampling four reflectance spectra with an Ocean optic USB 4000

spectrophotometer (Ocean Optics, Dunedin, FL) on five breast feathers per individuals lightened by a dual deuterium and halogen light source (Mikropackan DH-2000-BAL, Mikropack, Ostfildern, Germany). As in (Antoniazza et al. 2010), the individual mean brown chroma, which represents the contribution of the red part of the spectrum (600-700nm) to the whole spectrum (300-700nm), was calculated.

The individual breast-colour variation in the barn owl ranges from purely white to rufous-brown (dark). Because the genetic basis for this trait is still poorly known (Roulin & Dijkstra 2003), we investigated five alternative genetic architectures. (i) The simplest architecture consists of a single bi-allelic locus. More complex ones involved (ii) 25 bi-allelic loci; and (iii) a single multi-allelic locus with 50 serial alleles. For these three architectures, the determination of the colour phenotype was defined as purely additive (i.e. no dominance, nor epistasis). Additionally, we explored architectures with (iv) a single bi-allelic locus and (v) 25 bi-allelic loci, where the dark allele was completely recessive. Even though somewhat unrealistic, this dominance scheme was used in order to allow for a higher initial dark allele frequency in the refugium, while keeping the frequency of the dark phenotype at its observed value, which would facilitate the surfing phenomenon (Hofer et al. 2009). In other words, we chose this dominance scheme in order to be conservative, by favouring the neutral processes.

In the bi-allelic architectures (for either one locus or 25 loci), one allele was considered “white” (representing the whitest birds), the other “dark” (representing the darkest birds). In the multi-allelic architecture, alleles are distributed over a linear gradient ranging from “whitest” to “darkest” with 50 different levels. Also, as a control, we simulated 22 microsatellite loci to mimic the purely neutral markers used in the previous simulations.

As the initial frequency of a given allele (Hofer et al. 2009) or the geographic location where a new allele appears (Klopfstein et al. 2006; Travis et al. 2007) may play a major role in the probability of observing the surfing phenomenon, two models varying in these respects were designed: (i) evolution from standing variation and (ii) facilitated allele surfing. This second scenario was implemented only for the architectures without dominance, in order to estimate the probability of surfing for a new mutation occurring at the front of the expansion. For all these models, range expansion started from an Iberian refugium colonising the rest of the continent, potentially generating clines in colour polymorphism through the process of allele surfing.

Initial allele frequencies depended on the model used. For models based on standing variation, the average initial frequencies were calculated based on the current phenotype frequencies observed in the refugium of the Iberian Peninsula where the white phenotype is currently present at a ~90% frequency. Accordingly, for the co-dominance models, the initial frequency of the "white" allele was 90%; for the complete dominance models, it was 68%. In the multi-allelic model, the frequency of each allele was given by an exponential distribution, in which the lighter-coloured half of the alleles had a frequency of 90%.

For the simulations with facilitated allele surfing, the whole Iberian Peninsula started already occupied and the white allele was fixed in all patches. One patch, located in the north-eastern corner of the Iberian Peninsula, contained a single dark allele at each locus (also for the multi-locus architecture) and initial population size (Ni) determined by migration rate (m) and carrying capacity (K): $Ni = K \times m/4$. For the multi-allelic trait, the new mutation was implemented by bringing the darkest allele into the population which, in this case, contained the same exponential distribution of the other alleles as used in the evolution from standing variation scenario. As a result, this dark allele was at the very front

end of the expansion, giving it an enhanced chance to spread by hitchhiking on the colonisation wave and creating the observed cline.

Beyond dominance effects, the mapping of genotypes into phenotypes was also done considering two different values for heritability of the colour trait (h^2 = 0.81 or 1). These values were chosen because narrow-sense heritability for colour was estimated to be 0.81 in Switzerland (Roulin & Dijkstra 2003; Roulin et al. 1998), and complete heritability (h^2 = 1) makes the estimation of phenotypic differentiation (Q_{ST}) more conservative.

For all simulations, we calculated the linear regression between pairwise geographic distances and the neutral genetic (F_{ST}) or phenotypic differentiation (Q_{ST}) between the 20 sampled populations. To assess the steepness of the cline produced, we retained the slope of the linear regressions, and following (Antoniazza et al. 2010) used the difference in slope between Q_{ST} and F_{ST} as a statistic to summarize the discrepancy between phenotypic and neutral markers differentiation. Finally, we compared the values for the difference of slopes obtained in each one of the simulation models with the relative position of this statistic as calculated for the observation. The proportion of simulations in each model that returned values equal or higher than the observation provided us with an estimate of the probability of attaining the observed values with that given neutral model.

Results

Model comparison

The posterior probability of each of the eleven models tested is presented in Fig. 2. The four models with one glacial refugium are best supported and their total posterior probability is higher than 90%. Among the one-refugium models, the base scenario with a

constant carrying capacity over the continent had the highest posterior probability (0.31), followed by the model with a south-west/north-east cline in carrying capacities (0.25). The former model has not only higher support, but is also more parsimonious than the latter and was therefore used for all further simulations. Interestingly, the estimation of the parameters for the second best model, with a cline in carrying capacity, (although clearly less supported) results in estimates with a very shallow or non-existent cline of carrying capacities, thus equivalent to the simplest one-refugium one-carrying-capacity model. This also applies to the other two one-refugium models (see Fig. S5-7 for the parameter estimates).

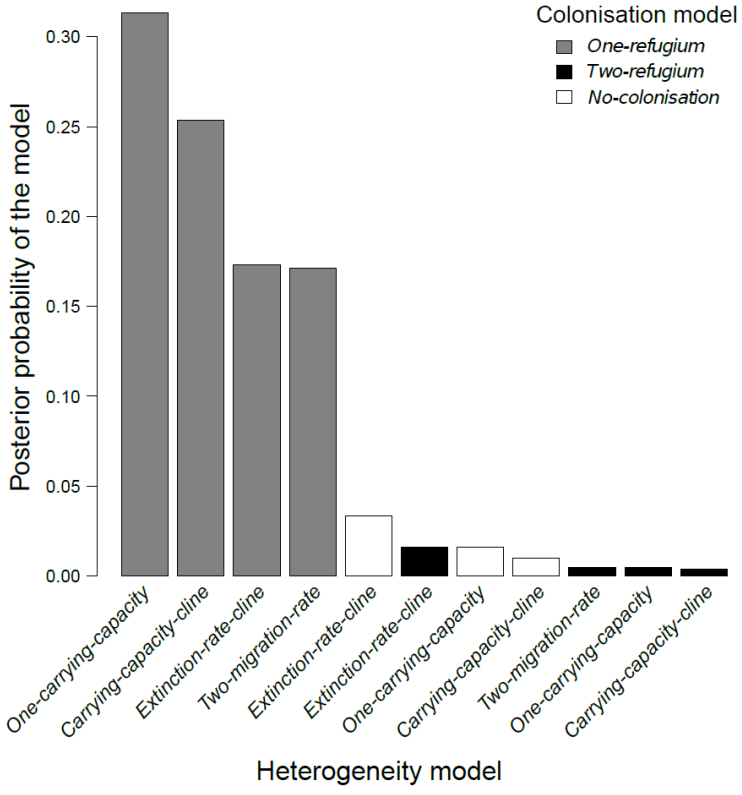

Figure 2. Posterior probabilities of the 11 models tested based on Leuenberger and Wegmann (2010). Based on four pattern statistics, 1000 simulations over 100 000 simulations per models were retained.

Demographic parameter estimates

The posterior distributions for the demographic and genetic parameters of the base model (one refugium with one carrying capacity) are shown in Fig. 3, and the corresponding point estimates are reported in Table 3. The carrying capacity shows a narrow posterior distribution with a mode at 203 individuals per deme and a HPD95% varying between 76.5 and 555. The population growth rate as well as the refugium population size show broad posterior distributions, and their point estimates of respectively 1.58 and 59 800 should be considered with caution given their low estimability (see below). Migration rate estimates show high values with a mode at 0.375 and an HPD95% of 0.188-0.5. The mutation rate showed a very narrow posterior distribution with a mode of 1.03×10^{-4} and a HPD95% between 2.85×10^{-5} and 3.8×10^{-4}. The estimation of the onset of colonisation indicates high values with a mode at 7350 generations, which corresponds to about 24 500 years BP according to the generation times estimated in a Swiss barn owl population (Altwegg et al. 2006) and its HPD95% varies between 3810 and 10 000 generations ago.

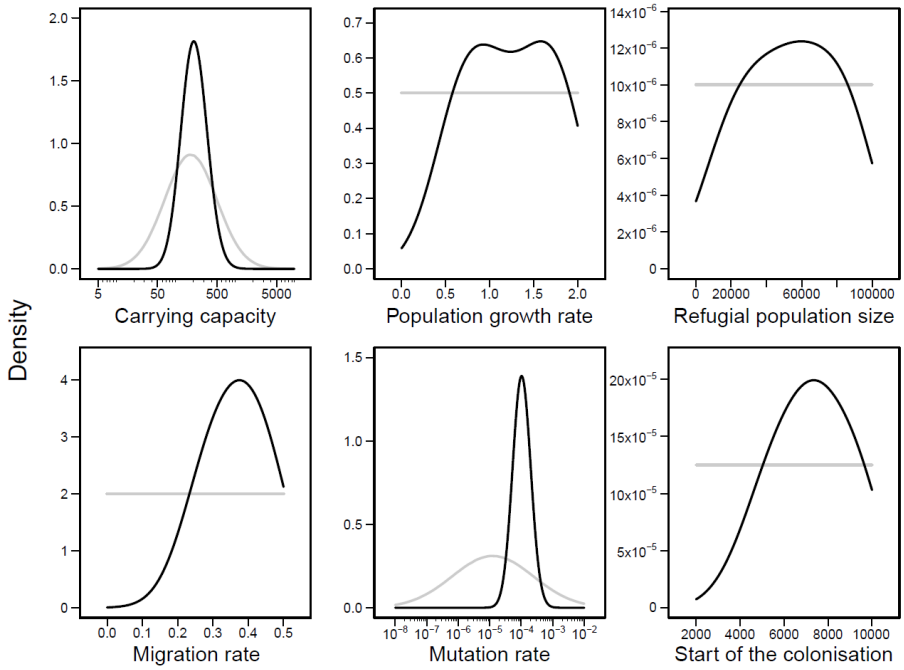

Figure 3. Posterior distributions of the estimated parameters. Grey lines show prior density and black lines the posterior distributions. Note that carrying capacity and mutation rate are in logarithmic scale. The same smoothing parameters were used for all parameter estimates (i.e. Dirac peak width = 0.02).

Table 3. Parameter estimates under the best supported model (one-refugium single carrying capacity). Estimated modes are used as point estimates; HPD95% stands for the 95% highest posterior density intervals.

Demographic parameters	Estimated modes	HPD95%
Start of the colonisation (generations)	7350	3810 - 10 000
Population growth rate	1.58	0.338 - 2.00
Mutation rate	1.03×10^{-4}	2.85×10^{-5} - 3.80×10^{-4}
Size of refugium population	59 800	8840 - 98 800
Migration rate	0.375	0.188 - 0.500
Carrying capacity	203	76.5 - 555

Quality assessment

All statistics that assess the quality of the estimation of the parameters (RRMSE, R^2, relative bias) are consistent with which parameters can be well estimated and which ones cannot (Table 4). The RRMSE of the parameter estimation varies widely from 0.0516 to 7.55. The estimate for mutation rate is highly accurate; those for migration rate, carrying capacity and start of the colonisation are also quite good. Population growth rate and refugium

population size are poorly estimated, which is not unexpected since these parameters have only an effect on the demographic history during a short period of the simulation (i.e. during the colonisation process). The validation analyses showed that our estimates are generally conservative: More pseudo-observed simulations are generally found in the posterior distribution than expected in general (Table 4), but for start of colonisation and the refugium population size. As these distributions were used as the background model for the colour simulations, we can be confident that they provided solid foundations.

Table 4. Validation of the estimates for the *one-refugium*, *one-carrying-capacity* model based on 1000 pseudo-observations. See Material & Methods, *Quality assessment of estimates* for more details.

Demographic parameters	Rel. bias	RRMSE	Prop. HPD 50%	Prop. HPD 95%	R^2
Start of colonisation	0.109	0.536	47	93.2	0.154
Population growth rate	0.744	5.9	52.6	94.9	0.0952
Mutation rate*	0.00336	0.0516	60.8	98.0	0.955
Refugium population size	1.24	7.55	48.9	94.7	0.0582
Migration rate	0.206	0.919	56.5	97.3	0.576
Carrying capacity*	0.00978	0.114	65.5	98.7	0.649

*As for the parameter estimate, these parameters are in log scale.

Colour simulations reveal adaptive origin of colour cline

The probability of generating the observed colour cline under a strictly neutral model was assessed with a second round of simulations. For each combination of genetic architecture and model of polymorphism distribution (dominance or not, facilitated allele surfing or not), we generated 1000 replicates. The results for the comparison between these simulations and the observed values are presented in Fig. 4. For the models based on standing variation, we observe that no simulation reached the observed values (with either h^2 = 1 or 0.81), no matter the dominance. When mutations are enforced to take place at the very front of the expansion (facilitated allele surfing), between one and five simulations

produced differences of slopes equal to or larger than what is observed in the owl populations (1 out of a 1000 for both one-locus traits, 3 and 5 out of 1000 for the multi-locus trait with the h^2 = 0.81 or h^2 = 1, respectively). In summary, without selection, very few simulations under an unlikely scenario managed to recreate the abrupt cline in colour visible in the observed data.

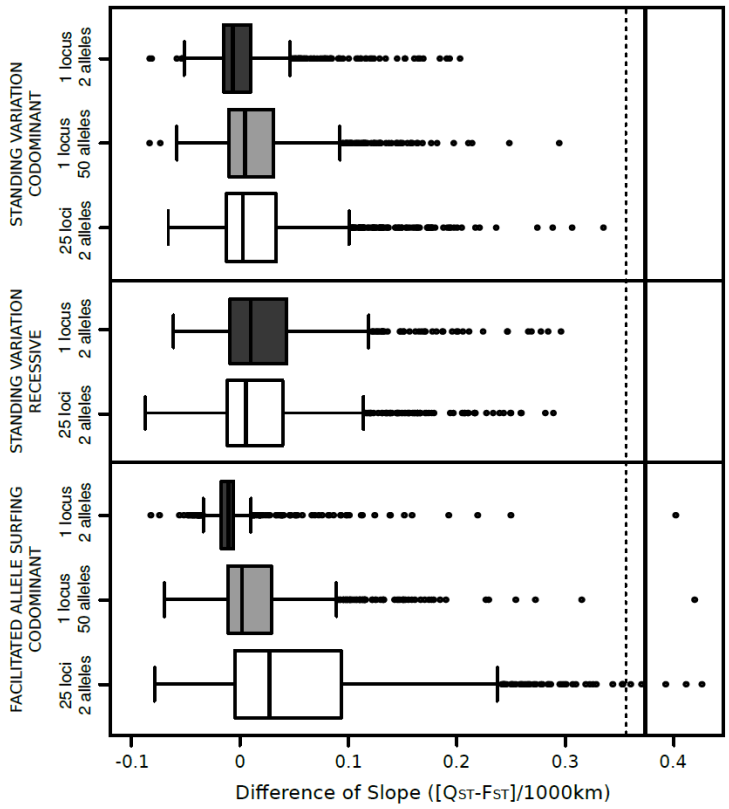

Figure 4. Probability of the neutral simulations to replicate the observed cline in colouration in the European barn owl. Comparison of distributions obtained from the calculation of the slope of IBD for the quantitative trait (colour, Q_{ST}) and the neutral loci (F_{ST}). Each model of different genetic architectures and starting polymorphisms is represented as a different distribution. The observed values for different levels of heritability are represented by the vertical lines: dashed line with h^2 = 1, plain line h^2 = 0.81.

Discussion

Neutral demographic model

The spatially explicit approximate Bayesian computation analysis strongly supported the hypothesis that barn owls colonised Europe after the LGM from a single refugium

situated on the Iberian Peninsula. It appears that the sequential bottlenecks during colonisation alone explain the pronounced continuous decrease in diversity from the Iberian Peninsula to Eastern Europe. Alternative models including additional processes capable to explain the observed cline (cline in carrying capacity, cline in extinction rate, or to the existence of two refugia) were less supported than the simpler model. Even more complex models ¬– including, for example, mountain ranges or variation in ecological suitability – might be more precise for some parts of the distribution of the European barn owl; but this general model provides a good approximation of the general picture. Another possible development of our model would be to allow for long-distance dispersal (LDD). However, we believe that – if LDD were a meaningful process in the whole scenario of the barn owl's colonisation of Europe – it would have left a different signature of isolation by distance, with a less consistent pattern than the one observed both in pairwise genetic distances between populations and in the decay of genetic diversity (Antoniazza et al. 2010). Furthermore, a long distance is determined by the scale of the model in use: The deme dimensions we implemented here represent the average dispersal distance of barn owls in nature (50km) (Bunn et al. 1982), which we believe is probably larger than the dispersal ability during the deglaciation period post-LGM. In summary, LDD may have played o role in the demographic history of *Tyto alba*, but not a role that was relevant in the colonisation process and, therefore, not a role that would alter the significance of our findings.

The estimated start of the colonisation of 7350 generations ago with a 95% confidence interval of 3810 to 10 000 generations ago corresponds to 26 460 years BP and a 95% confidence interval from 36 000 to 13 700 year BP (assuming the estimated 3.6 years per generation, Altwegg et al. 2006) falls in line with an expansion following the LGM. This estimate is, although slightly higher, in good agreement with the expected time of

colonisation of Europe after the LGM 20 000 years BP. The estimated time of colonisation in years BP depends highly on the generation time, which is difficult to estimate and also assumes that the generation time remains constant over time. The generation time estimated by Altwegg et al. (2006) came from short interval of time in a stable population. Given the ability of Barn owls to already reproduce at one year of age (Cramp 1985) and that this age at first breeding is the parameter that matter in a range expansion scenario; it is very likely that the generation time measured by Altwegg et al. (2006) is higher than the actual generation time over the post-glacial history of European barn owls. This could explain the relatively old estimates of the colonisation time by the barn owls.

The estimated carrying capacity of ~200 individuals per deme with a 95% confidence interval of 77 to 555 individuals extrapolated to the European scale results in a population size of 542 000 with a confidence interval of 204 300 to 1 482 400. Compared to the estimate of 140 000 breeding pairs (Hagemeijer & Blair 1997), i.e. 280 000 breeding individuals in Europe this seems to be overestimated. Even if these two numbers cannot be compared directly (the first one is an effective population size and the second one a census size for the breeding adults), they are in the same order of magnitude and our estimate is plausible. The overestimation is probably also based on the fact that the simulated European map is slightly larger than the actual natural range of barn owls.

The estimated migration rate of 0.375 between neighbouring demes of 50 km × 50 km is in accordance with what was estimated by Bunn et al. (1982) for the Netherlands (32.1 % of the young move more than 50 km in their first year). The estimated size of the refugium population of 59 800 individuals has to be taken with caution. As expected the accuracy tests show that this parameter is difficult to estimate since its traces in the genetic diversity in the present is secondary. In contrast, the estimate of the mutation rate is very accurate, with an

estimate of 1.03×10^{-4} and a sharp 95% confidence interval from 2.85×10^{-5} to 3.80×10^{-4}. This estimate is in good agreement with the expectation (see discussion in Wegmann & Excoffier 2010). These estimates seem to be biologically meaningful and we are thus confident that this demographic and genetic model is a good approximation of the actual post-glacial history of the European barn owls and that it provides a sound demographic null model to investigate further questions regarding barn owl biology.

Colour simulations

Our finding that Europe was colonised from a single Iberian refugium has the implication that the colour cline (Fig. S4) might have been established by surfing during this colonisation. Simulations of a colour quantitative trait in our neutral demographic model were run in order to evaluate this possibility. Overall, the formation of the observed cline under neutrality is extremely unlikely: We seldom obtained simulations showing the same strong difference in geographic differentiation between phenotype and neutral markers. We never observed it with standing genetic variation in the refugium. Only with facilitated allele surfing (i.e. explicitly seeding mutations in the front of expansion) did we obtain between 1 and 5 simulations (out of a 1000) showing the same or larger differences. The highest number of such simulations was obtained for the trait architecture based on 25 bi-allelic loci (and hence, 25 mutations, 1 per locus, in the deme at the start of the expansion), but even with this unrealistically favourable architecture the probability of generating by neutral processes only such a difference in slope was less than 0.5%.

The colour simulations thus show that the evolution of the colour cline by surfing is almost impossible. While we could not be exhaustive in our tests of alternative genetic

architectures, we believe that the architecture where the surfing mutation is recessive and therefore present at a fairly high frequency is the most favourable for surfing. Under this architecture, surfing did not happen. Architectures with epistasis were not investigated but would also need one allele to be driven to a high frequency. Furthermore, the current knowledge on the typical architectures underlying pigmentation traits in birds suggests that a rather simple architecture is to be expected in the case of the barn owl (Mundy 2006; Roulin & Ducrest 2013). Therefore, the conclusion drawn by Antoniazza et al. (2010), that the European colour cline results from a local adaptation process, is thus confirmed by our simulation approach. With the exclusion of neutral scenarios, the evolution of the colour cline by natural selection generating local adaptation is indeed far more likely.

Evolution and maintenance of the European barn owl colour cline

The classical view on the evolution of barn owl colour variation in Europe is that the colour morphs evolved in allopatry in two refugia during the last glaciations and that the cline evolved by secondary contact after the ice age (Voous 1950). The model inferred above for the post-glacial history of the barn owl in Europe points toward a very different scenario. Our results suggest that the colour cline evolved during or after the colonisation out of a single refugium through a local adaptation process and also imply a very recent evolution of the colour cline (post-glacial, hence younger than 20 000 years BP). A rapid colonisation of Europe after the last ice age is supported by the observation of barn owl remains that dated at least from 10 000 years BP found in the UK (Del Hoyo et al. 2000; Yalden & Albarella 2009) and the estimated onset of colonisation of 7349 generation points toward a colonisation date close to the end of the last glacial maximum. Evaluating the strength of selection will be

a next step to further the understanding of this system, but the lack of information on the selective agent behind the colour variation puts a serious challenge to this extension (Antoniazza et al. 2010).

Continental clines and evolution during range expansions

We feel that the case of the barn owl, where evolution of a locally adapted trait happened during or after the recolonisation of the continent after the ice age, might be far more common than currently recognized in other taxa. The climatic oscillations of the quaternary that shape the dynamics of the ranges of many species of temperate latitudes on both hemispheres, generated retreat/recolonisation cycles that occurred along major climatic axis (mainly north-south). Also, there is a growing body of evidence that local adaptation along such climatic axes is rather common [for instance, size clines first describe by Bergmann (1847)].

Continental clines in temperate latitude thus offer a scope to study both local adaptation at large scale, but also the dynamics of this adaption in time and its interaction with colonisation processes. The interaction between natural selection and colonisation processes is a key question in evolutionary biology, but is still in its infancy (Excoffier et al. 2009). The study of large scale continental gradient might represent a fruitful area to study these questions in more details (see Kujala & Savolainen 2012 for a first approach with a non-spatial demographic model).

The European barn owl is a good illustration and provides a superb case study to investigate these questions. Here, we were able to show that the colour cline observed in this species was not established by neutral demographic processes during the colonisation of

the European continent. This shows that selection processes must have been involved in the establishment of the European colour cline, even if the mechanism by which these colour clines established remain to be elucidated. We believe that the demographic model developed in this study provides a sound historical scenario to further decipher adaptive and non-adaptive processes in this and potentially other species.

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Supplementary information

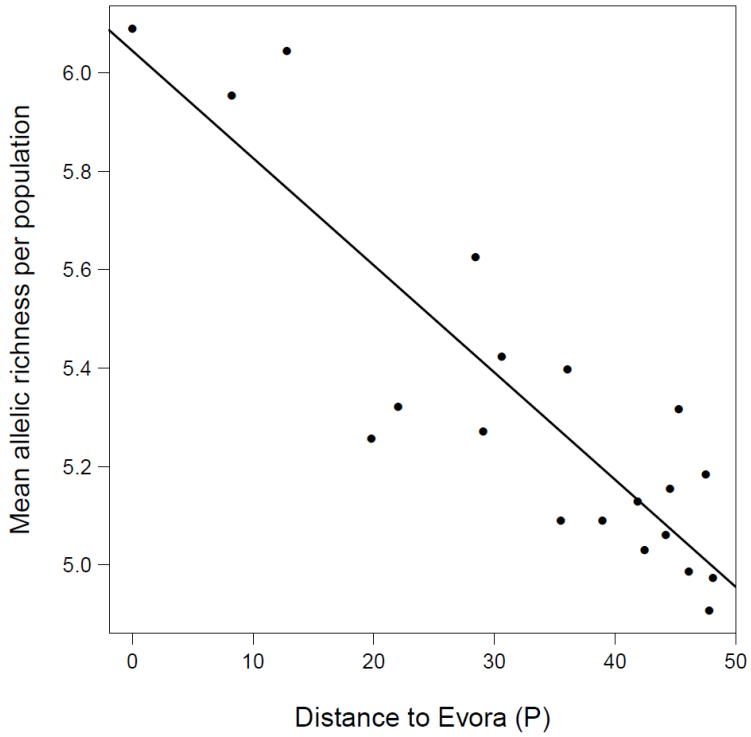

Figure S1. Observed cline in mean allelic richness (mean allelic richness over 22 loci per population as a function of distance to the south-westernmost population). Note that distances are in deme units (50 km). R^2 = 0.779.

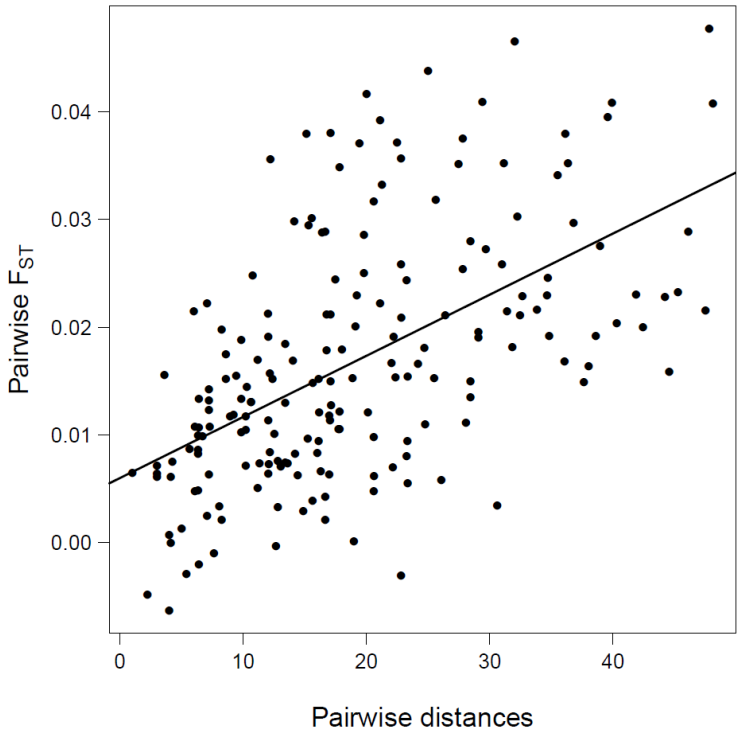

Figure S2. Observed isolation by distance (pairwise F_{ST} as a function of pairwise distances). Note that pairwise distances are in deme units (50 km). R^2 = 0.310.

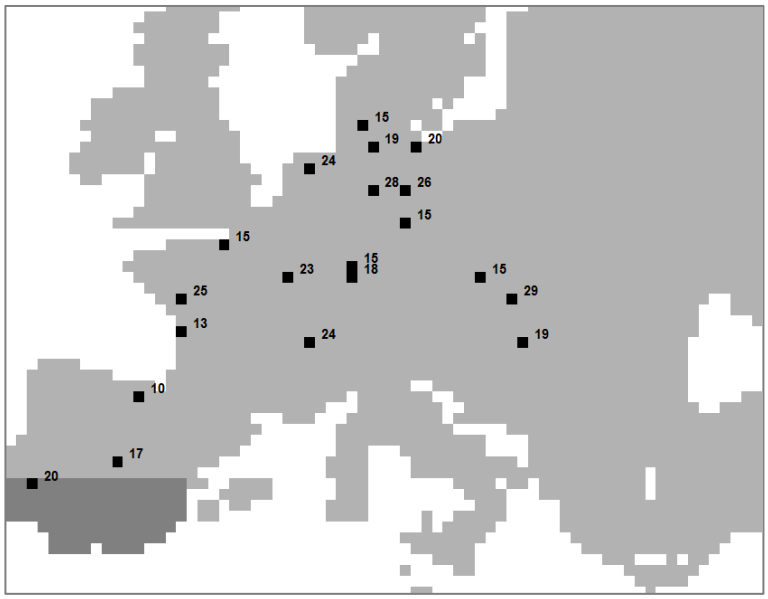

Figure S3. Simulated map of the sampling locations and sampling sizes. Sampling sizes and sampling locations as for the observed dataset are indicated. Similar sampling locations and sampling sizes are generated for the simulated dataset. Colonisable demes are indicated in grey, sea demes (not colonisable) are indicated in white. The Iberian glacial refugium demes are indicated in dark grey. We use a Europe Albers Equal Area Conic projection to adequately represent surfaces.

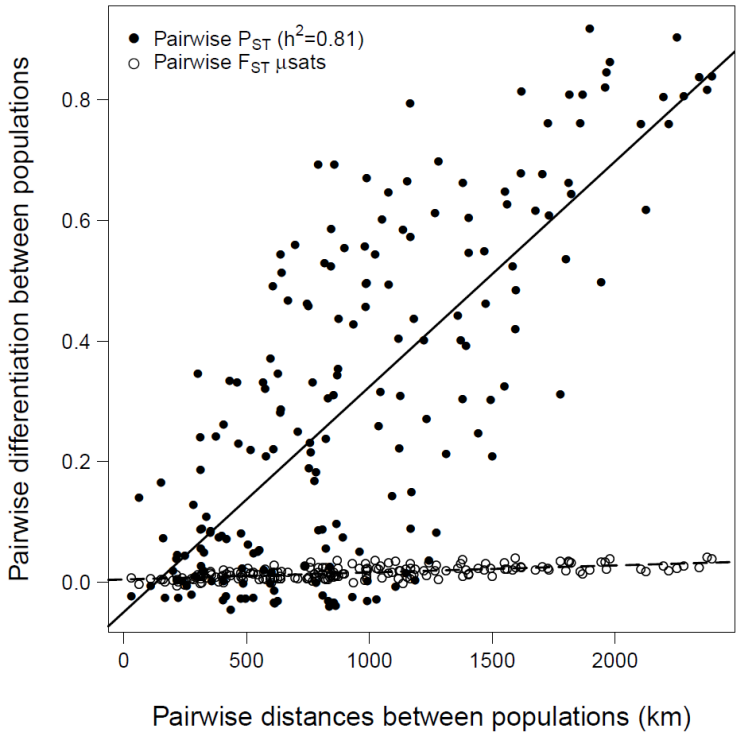

Figure S4. Observed isolation by distance for the microsatellites (pairwise F_{ST} as a function of pairwise distances, same data as in Fig. S1) and for the colour data (pairwise P_{ST} as a function of pairwise distances). Note that distances are in kilometres. This figure is an update (more microsatellite markers and more individuals) of bottom left panel of figure 2 in the first chapter of this thesis.

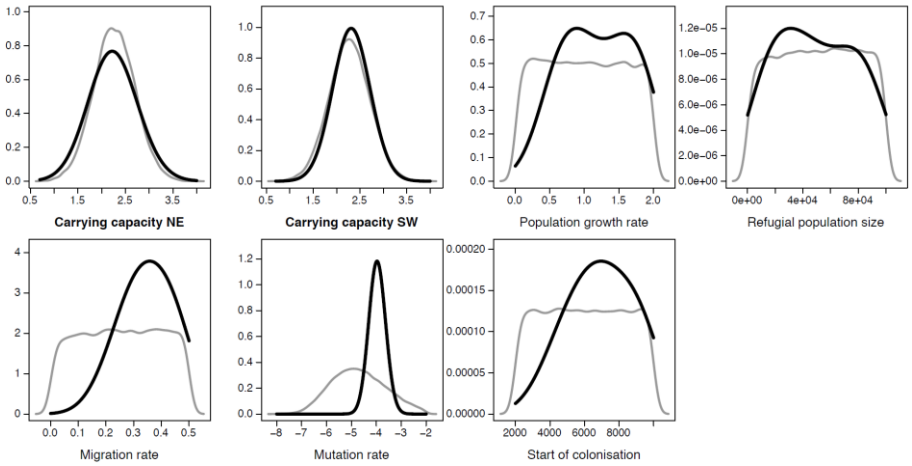

Figure S5. Posterior distributions of the estimated parameters for the *one-refugium carrying-capacity-cline* model based on 1000 retained simulations over ~10^6 simulations. Grey lines show prior density and black lines the posterior distributions. Note that carrying capacity and mutation rate are in logarithmic scale. The same smoothing parameters were used for all parameter estimates (i.e. Dirac peak width = 0.02). The estimated modes for the carrying capacities of the south-west and north-east corner of the map are very similar (205 vs. 166 respectively with massive overlap of the posterior distributions). The estimated model is thus very similar to the *one-refugium one-carrying-capacity* model and the difference in carrying capacity is in the expected direction.

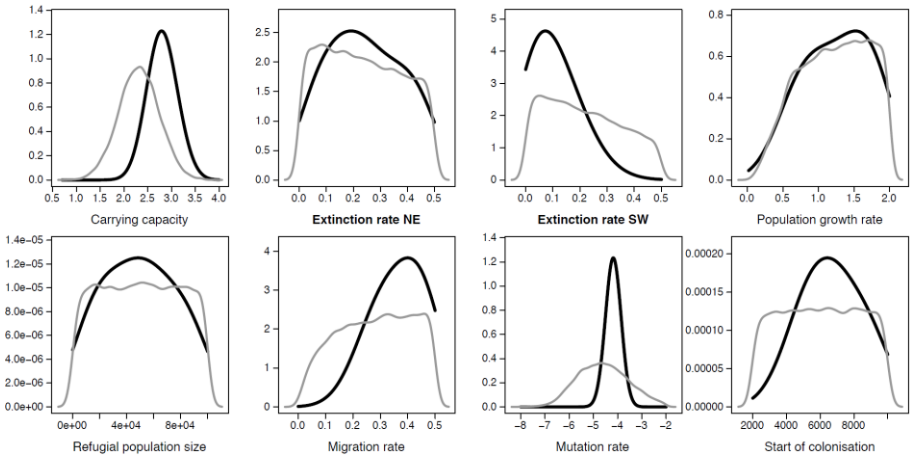

Figure S6. Posterior distributions of the estimated parameters for the *one-refugium extinction-rate-cline* model based on 1000 retained simulations over ~10^6 simulations. Grey lines show prior density and black lines the posterior distributions. Note that carrying capacity and mutation rate are in logarithmic scale. The same smoothing parameters were used for all parameter estimates (i.e. Dirac peak width = 0.02). Both estimates for extinction rate fall in the lower part of the prior distribution, especially the one in the south-west, which would have a strong effect on the fate of the simulations.

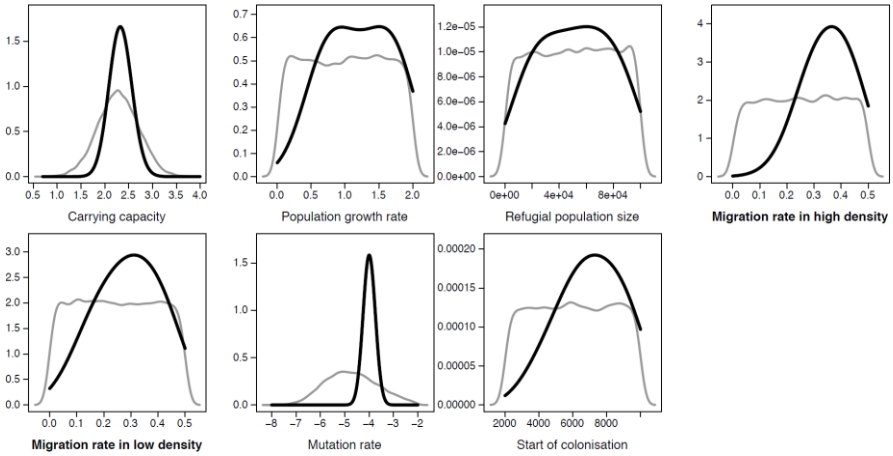

Figure S7. Posterior distributions of the estimated parameters for the *one-refugium two-migration-rate* model based on 1000 retained simulations over ~10^6 simulations. Grey lines show prior density and black lines the realised distributions. Note that carrying capacity and mutation rate are in logarithmic scale. The same smoothing parameters were used for all parameter estimates (i.e. Dirac peak width = 0.02). The posterior estimates for the two migration rates are very similar. It is interesting to note that mode of the current migration rate is somehow higher than the one during colonisation, which might be constrained by the low genetic structure observed in the barn owl today.

Table S1. Multiplex composition and primer concentration for microsatellite genotyping.

Multiplex	Locus	Dye	Final Conc. [µM]
Multiplex 1	Ta-206	FAM	0.45
	Ta-210	HEX	0.105
	Ta-216	FAM	0.135
	Ta-306	NED	0.165
Multiplex 2	Ta-218	HEX	0.178
	Ta-220	FAM	0.11
Multiplex 3	Ta-204	HEX	0.25
	Ta-214	FAM	0.5
	Ta-305	FAM	0.5
	Ta-310	NED	0.25
	Ta-413	NED	0.25
Multiplex 4	Ta-202	FAM	0.25
	Ta-212	DYO630	1
	Ta-215	FAM	1
	Ta-402	NED	0.25
	Ta-408	HEX	0.5
Multiplex 5	FEPO42	FAM	0.24
	54f2	NED	0.24
	Tgu06	HEX	0.48
	Calex-05	DYO630	0.48
	RBG18	FAM	0.72
	Oe053	HEX	0.96

Primer concentration is indicated for both forward and reverse primer together.

Chapter 3: Once around the Mediterranean: Color-related incipient ring speciation in European barn owls

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Key words: ecological speciation, local adaptation, melanin-based coloration, *MC1R.*

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Summary

Unraveling how neutral and adaptive evolution contribute to the build-up of reproductive isolation is key to advance our understanding of speciation. Species with a ring-like colonization around a geographic barrier in which reproductive isolation evolved in terminal populations offer unique insights, as intermediate stages of speciation can be observed across the ring. Here we present evidence for incipient speciation in European barn owls (*Tyto alba*) in a ring around the Mediterranean. From the Middle East barn owls colonized the Palearctic in two directions. A major colonization advanced over North Africa and Iberia, from where it colonized Europe and reached the secondary contact zone in Greece. A second colonization front arrived in Greece over the Bosporus. The colonization of Central Europe was accompanied by the evolution of a novel rufous phenotype caused by a non-synonymous derived variant of the melanocortin-1- receptor (*MC1R*) gene. Steep geographic clines in coloration and in the frequency of the novel *MC1R* variant are maintained by local adaptation, despite weak neutral genetic population structure. Admixture patterns and linkage disequilibrium of coloration with the neutral genetic background in the secondary contact zone suggests that introgression is limited among the terminal forms of the ring. The evolution of a novel color phenotype during the ring-like colonization of barn owls resulted in color-related limitation of introgression at secondary contact. This shows how derived genetic variation can contribute to the evolution of new phenotypes and may ultimately lead to the evolution of reproductive isolation in the face of ongoing gene flow.

Introduction

The 'Origin of Species' established adaptation by natural selection as the foremost process by which species evolve. Ever since, the relative contributions of adaptive and neutral evolution in species diversification have been debated. For decades, the prevailing view held that reproductive isolation evolves in geographic isolation, and emphasized the need for a period with restricted gene flow during which incompatibilities not necessarily evolve by local adaptation, but are established by neutral genetic drift (Bolnick & Fitzpatrick 2007; Mayr 1963). Today an increasing body of theoretical and empirical work shows that under strong ecology-driven divergent selection populations can diverge and species evolve in the presence of gene flow (Coyne & Orr 2004; Nosil 2008; Via 2001). Allopatric speciation and speciation-with-gene flow represent two extremes of the speciation process in which either genetic drift or selection predominate (Fitzpatrick et al. 2008). However, species may rather evolve by a complex interaction of demographic effects and local adaptation. Insights into the interplay of demography, geography and ecology during species evolution are therefore key to understanding how biodiversity unfolds.

Ring species represent ideal systems to study the interplay of demography and selection during the evolution of reproductive isolation. They consist of populations with a ring-like distribution around a geographic barrier – such as mountain ranges (Irwin et al. 2001b), valleys (Kuchta et al. 2009), or a sea (Bensch et al. 2009) – which vary gradually in one or several phenotypic traits, and the populations at the ends of the ring meeting in secondary contact are reproductively isolated (Irwin et al. 2001a). Within such systems, reproductive isolation is predicted to evolve through the interaction of drift during colonization and local adaptation to the environments through which the colonization fronts advance (Doebeli & Dieckmann 2003; Martins et al. 2013). During this process populations

remain interconnected by gene flow throughout the ring. Ultimately, reproductive isolation is revealed only at secondary contact, where the colonization fronts have accumulated too many genetic and/or phenotypic differences to successfully interbreed. Despite a high, world-wide abundance of geographic barriers that could give rise to ring species (Monahan et al. 2012), ring speciation has only rarely been documented (Irwin et al. 2001a). The scarcity of the phenomenon is likely explained by the narrow demographic conditions under which ring species evolve. For reproductive isolation among terminal forms to evolve, the rate of diversification by genetic drift and divergent selection must outweigh the homogenizing force of gene flow (Irwin et al. 2001a). Conversely, the ring may break up into multiple taxa if diversification occurs too fast (Doebeli & Dieckmann 2003). Accordingly, ring species known to date exhibit varying degrees of phenotypic and genetic differentiation. *Ensantina* salamanders (Pereira & Wake 2009), Greenish warblers (*Phylloscopus trochiloides*, Irwin et al. 2001b), and Australian *Platycercus* parrots (Joseph et al. 2008) show little or limited gene flow among populations and complete cessation of gene flow between the terminal taxa. The opposite extreme, with high rates of gene flow across a ring around the Baltic Sea, is found in the willow warbler (*Phylloscopus trochilus*, Bensch et al. 2009).

With a zone of secondary contact in which the two colonization fronts meet, and gradual genetic and phenotypic changes in-between, ring species join two systems to study speciation: they provide access to a hybrid zone in which incompatibilities can be mapped, while the evolutionary history of the reproductive barriers can be studied in the phenotypic and genetic cline between the reproductively isolated terminal taxa. Here, based on genetic data from 22 microsatellite markers and a mitochondrial marker (NADH-dehydrogenase-6 gene, *ND6*) together with data on melanin-based coloration and from a candidate color gene (melanocortin-1-receptor, *MC1R*) in an extensive sampling of >700 barn owls (*Tyto alba*)

from 28 populations from Europe, North Africa and the Middle East (Figure 1A, Table S1), we provide evidence for circum-mediterranean ring speciation of barn owls, and link limited introgression at secondary contact with the adaptive evolution of a novel color phenotype that arose during colonization.

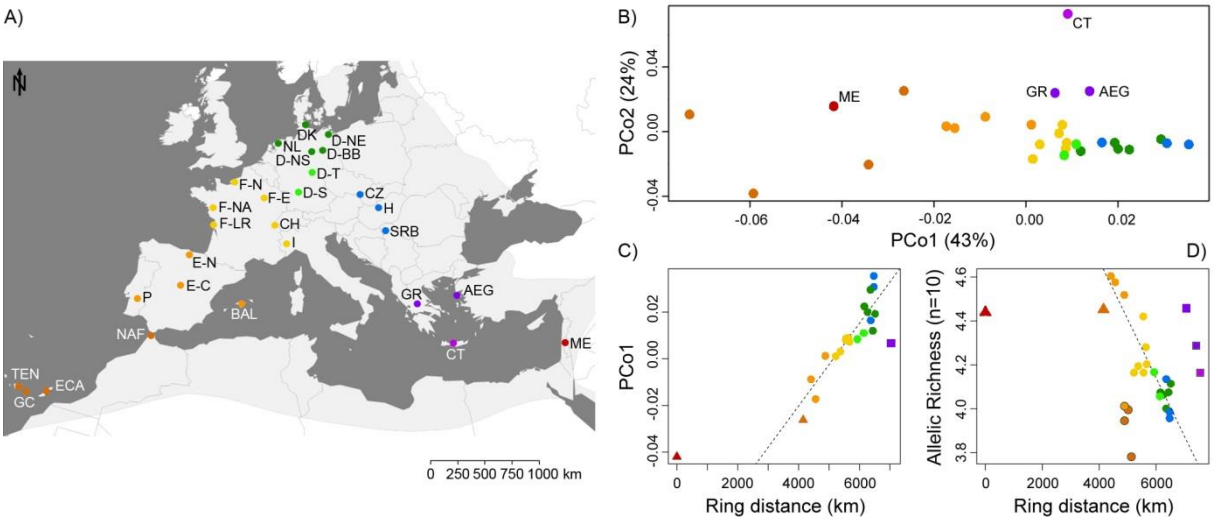

Figure 1. Sampling and population structure. **A)** Geographic distribution of sampling locations of barn owls. The distribution of barn owls in the study range is shaded in light grey. *AEG*; Aegean (N=22); *BAL*, Baleares (N=29); *CH*, Switzerland (N=27); *CT*, Crete (N=61); *CZ*, Czech Republic (N=20); *D-BB*, Germany Brandenburg (N=27); *D-NE*, Germany Northeast (N=21); *D-NS*, Germany Niedersachsen (N=30); *D-S*, Germany South (N=37); *D-T*, Germany Thüringen (N=19); *DK*, Denmark (N=37); *E-C*, Spain Center (N=20); *E-N*, Spain North (N=11); *F-E*, France East (N=28); *F-LR*, France La Rochelle (N=13); *F-N*, France North (N=15); *F-NA*, France Nantes (N=28); *GC*, Gran Canaria (N=16); *GR*, Greece (N=24); *H*, Hungary (N=32); *I*, Italy (N=25); *ME*, Middle East (N=32); *NAF*, North Africa (N=19); *NL*, Netherlands (N=30); *P*, Portugal (N=30); *SRB*, Balkans (N=28); *TEN*, Tenerife (N=26); *ECA*, Eastern Canaries (Lanzarote, Fuerteventura) (N=17). **B)** PCoA based on nuclear markers. Coloration follows A). **C)** Correlation of the first PCo axis of mainland populations with ring distance from the Middle East. The regression line is based on all European populations but Greece. **D)** Geographic structure of nuclear allelic richness. The regression line is based on all European mainland populations. C) and D): Greece, the Aegean and Crete are depicted with squares, Middle Eastern and North African populations with triangles, islands in D) with a black border.

Results

Ring-like population structure around the Mediterranean

Genetic differentiation among barn owl populations follows a shallow pattern of isolation-by-distance (overall F_{ST}: microsatellites, 0.045; *ND6*, 0.134) (Table 1, Figure S1). Strongest differentiation on the mainland was identified between the populations in Eastern

Europe and the Middle East, discordant with these population's spatial proximity (nuclear F_{ST}: Hungary-Middle East, 0.085; Balkans-Middle East, 0.084; Figure S2A). Principal Coordinate Analyses (PCoA) showed the same close relationship of the Middle Eastern population with the geographically distant populations from the Canary Islands, North Africa, and Iberia, while placing it most distant from the geographically closer populations in Eastern Europe (Figures 1B, S3), suggesting ring-like colonization around the Mediterranean. This hypothesis is consistent with the geographic distribution of admixture proportions estimated in Bayesian analyses of population structure (Pritchard et al. 2000) (Figures 2A, 2B). These analyses found highest support for two clusters among mainland populations ($\Delta K(2)=251$, Figures S4A-D), and for two and three clusters with islands included ($\Delta K(2)= 237$, $\Delta K(3)=331$, Figures S4E-H). Individuals from the Middle East, North Africa, the Canary Islands, and Crete were predominantly assigned to a "southern" lineage, while populations from Central, Northern, and Eastern Europe formed a "northeastern" lineage (K=2; Figures 2A, 2B). Populations from Iberia to Central Europe showed a gradual change from southern to northeastern ancestry (Figures 2A, 2B). The island population from Crete was split off from the southern lineage into its own cluster at K=3.

Table 1. Mantel r for tests comparing classical isolation-by-distance and ring-colonization hypotheses. In Mantel tests and partial Mantel test taking into account flight distance insularity was taken into account when island populations were included. P-values are provided in parentheses. Flight distance is denoted "Flight", shortest overland distance "Land", and ring distance "Ring". Values for models explaining most of the variance are provided in bold.

	Mantel tests						Partial Mantel tests			
	Islands included			Mainland			Islands included		Mainland	
	Flight	Land	Ring	Flight	Land	Ring	Land	Ring	Land	Ring
Msat	0.616 (0.001)	0.616 (0.001)	**0.674** (0.001)	0.751 (0.001)	0.777 (0.001)	**0.845** (0.001)	0.079 (0.170)	**0.381** (0.002)	0.326 (0.071)	**0.590** (0.001)
ND6	0.441 (0.001)	0.461 (0.001)	**0.544** (0.001)	0.620 (0.001)	0.632 (0.001)	**0.722** (0.001)	0.161 (0.059)	**0.355** (0.001)	0.159 (0.120)	**0.471** (0.013)
MC1R	**0.255** (0.004)	0.184 (0.019)	0.168 (0.061)	**0.307** (0.001)	0.253 (0.003)	0.250 (0.002)	-0.349 (1)	-0.064 (0.781)	-0.324 (1)	-0.029 (0.655)
Color	**0.199** (0.010)	0.126 (0.066)	0.109 (0.119)	**0.356** (0.002)	0.319 (0.001)	0.265 (0.002)	-0.361 (1)	-0.088 (0.812)	-0.340 (1)	-0.122 (0.902)

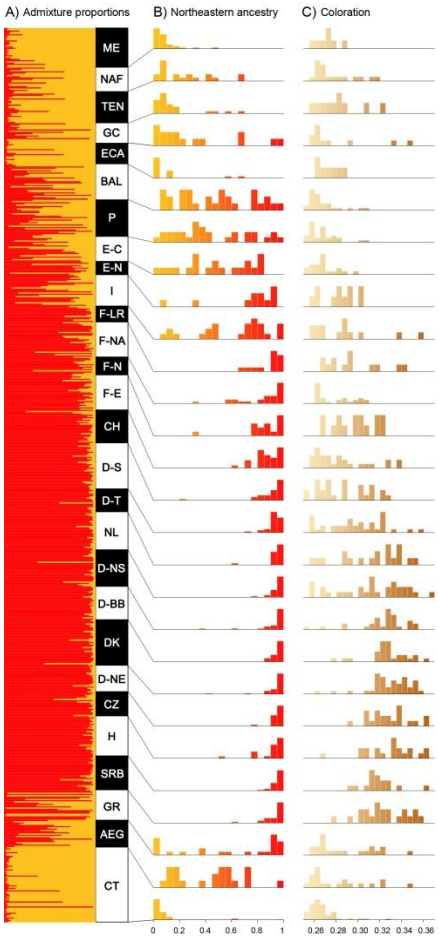

Figure 2. Admixture and color distribution per barn owl population. **A)** STRUCTURE barplot. Each horizontal line represents one individual. Proportions to which each individual was assigned to the southern (orange) and north-eastern (red) cluster are depicted. **B)** Population-wise frequencies at which individuals were assigned to the southern (left, orange shading) and northeastern (right, red shading) clusters. **C)** Population-wise color distribution (brown chroma). Population abbreviations are provided in Figure 1.

To corroborate the evidence for ring-colonization around the Mediterranean we performed tests that contrast alternative colonization hypotheses. Classical hypotheses of isolation-by-distance were modelled using distance matrices representing (i) flight distance, and (ii) shortest overland distance; ring colonization was modelled using (iii) ring distance, i.e. distance within a clockwise ring starting in the Middle East, through North Africa, Iberia, and Europe, and ending in Crete (see Figure S5), following PCoA results (Figure 1B, S3). Two methods that compared these models unequivocally supported the ring-colonization scenario (Tables 1, 2). In linear models that related flight, overland, and ring distance from the Middle East to genetic structure measured by the first PCo axis (PCo1), ring distance explained the highest amount of genetic structure (R^2=0.74, Table 2, see also Figure 1C). Flight distance best explained the genetic structure along PCo2 (R^2=0.40, Table 2), indicating genetic exchange among spatially close populations following colonization. In Mantel tests pairwise flight, overland, and ring distances all significantly correlated with genetic differentiation (Table 1, Figure S1). However, ring distance explained most of the variance (Mantel r=0.674, Table 1), also when accounting for dispersal (flight distance) among geographically close populations unrelated to colonization history (Mantel r=0.381, Table 1). Results were concordant between nuclear microsatellites and mitochondrial data, and not sensitive to the inclusion or exclusion of island populations (Tables 1, 2).

Table 2. Correlations of Principle Coordinate axes with geographic variables in barn owls. Flight distance is denoted "Flight", shortest overland distance "Land", and ring distance "Ring". Values for models explaining most of the variance are provided in bold.

Marker	Distance	PCoA 1				PCoA 2			
		Isl. included*		Mainland		Isl. included*		Mainland	
		p	R^2	p	R^2	p	R^2	p	R^2
Msat	Flight	0.034 *	0.41	0.351	0.04	$<10^{-3}$ *	**0.40**	0.040	0.20
	Land	0.043 *	0.40	0.476	0.03	0.003*	0.34	0.075	0.15
	Ring	$<10^{-6}$ *	**0.74**	$<10^{-6}$ *	**0.76**	0.825	0.05	0.748	0.01
ND6	Flight	0.169	0.23	0.269	0.06	0.815	0.01	0.394	0.04
	Land	0.140	0.24	0.360	0.04	0.638	0.02	0.366	0.04
	Ring	$<10^{-4}$ *	**0.58**	$<10^{-4}$ *	**0.59**	0.561	0.02	0.218	0.07

* Insularity included as factor

Genetic diversity and origin of the ring colonization

Colonization is expected to leave traces in the geographic distribution of neutral genetic diversity. Highest diversity was found in Southern Iberian populations (nuclear allelic richness, AR: Portugal, 4.60, Central Spain, 4.58) followed by Italy, Northern Spain, North Africa and the Middle East (Figure 1D). Lowest diversity on the mainland was found in Eastern Europe (nuclear AR: Hungary, 3.96; Balkans, 3.98; Brandenburg, 4.00) (Figure 1D). Diversity is lower on islands (islands: median AR=4.00, range 3.78-4.28; mainland: median AR=4.17, range 3.96-4.60) and decreases eastwards in the north of the Mediterranean (latitude x longitude: t=-3.84, $p<10^{-3}$; insularity: t=3.17, p=0.004; longitude: t=3.94, $p<10^{-3}$; latitude: -1.24, p=0.23; R2=0.64). Diversity also showed a strong tendency to decrease with increasing ring distance (islands included: insularity: t=-2.13, p=0.043, R2=0.28; ring distance t=-1.80, p=0.084; mainland populations: t=-2.97, p=0.008, R^2=0.31, Figure 1D) but no relation to flight or shorter overland distance. Within the European continent, a strong, significant decrease of diversity with distance from the Middle East was observed, with ring distance explaining most of the variance (ring distance: t=-8.73, $p<10^{-6}$, R^2=0.82; flight:

t=3.87, p=0.001, R^2=0.47; overland: t=5.00, $p<10^{-3}$, R2=0.60) (Figure 1D). The same relationship was found for mitochondrial diversity (t=-2.24, p=0.038, R^2=0.22).

To infer the population with allele frequencies closest to the ancestral population, we introduced outgroup populations from California, USA (*T. a. pratincola*), Singapore (*T. a. javanica*), and Australia (*T. a. delicatula*) into analyses of population structure. Both a dendrogram depicting relationships among the clusters inferred by Bayesian analyses, and a neighbour-joining tree based on pairwise F_{ST} between populations placed the populations from the Middle East at the root of the circum-mediterranean colonization (Figure S6, Text S1).

It thus appears that out of the Middle East barn owls first colonized the region south of the Mediterranean, and later spread through Europe in a more recent expansion from Iberia (Figure 3, Text S2).

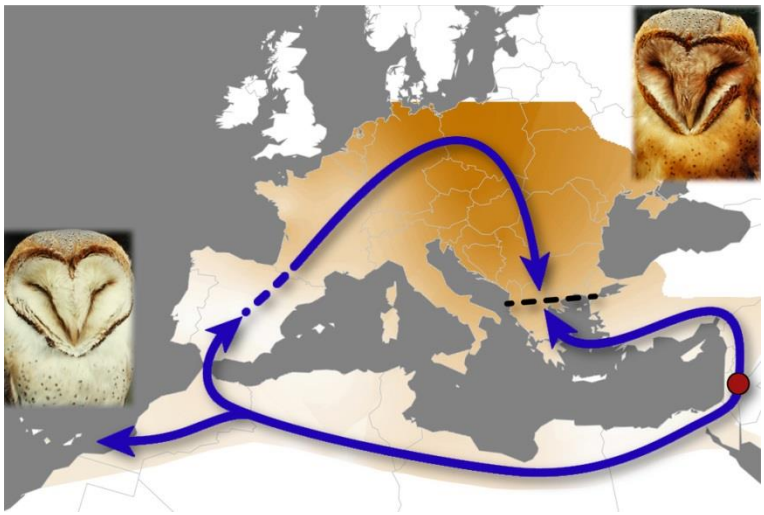

Figure 3. Circum-mediterranean ring colonization of European barn owls. The putative region of origin of the colonization is marked in red. Arrows indicate the colonization routes, the black dashed line the region of secondary contact with limited introgression at nuclear microsatellites. The color gradient (interpolated using the Kriging algorithm) and two typical white and rufous phenotypes are shown.

Secondary contact zone in Southeastern Europe

The above results place the populations from Eastern Europe and the Middle East, respectively, at the ends of a genetic continuum. The geographic region in-between therefore likely holds a zone of secondary contact. In line with this hypothesis, populations from Greece, the Aegean, and Crete exhibit peculiar genetic compositions reminiscent of hybrid zones: (i) Microsatellite data placed these three populations at intermediate positions in PCoA (Figures 1B, S2A), rather than with the geographically closest populations from Eastern Europe (and so did also mitochondrial data for Greece and the Aegean; Figure S3). (ii) The Greek population neither fits the otherwise strong correlation of mainland population structure with ring distance (Figure 1C); and (iii) for Crete, relationships were discordant between microsatellites (Figures 1B, 2A) and mitochondrial *ND6* (Figure S3).

Results from the above Bayesian clustering analyses were used to obtain further insights into patterns of admixture in Greece, the Aegean, and Crete. All Cretan individuals but 4 out of 61 (putatively one migrant, two F1 hybrids and one first-generation backcross) showed southern ancestry (Figures 2A, 2B). In contrast, the prevalence of predominantly Northeastern European haplotypes at mitochondria (Figures S7, S8) placed Crete unambiguously with Eastern European populations, a pattern also found for one out of 22 microsatellites (Ta-220). The presence of northeastern mitochondrial variation on a predominantly southern nuclear background provides evidence for a southern origin of Cretan barn owls, with mitochondrial introgression from the north and more limited introgression at the nuclear level.

The Greek and Aegean populations showed a fundamentally different genetic composition than the geographically close Cretan population. Different from all other populations, individual-level admixture appeared restricted: many individuals had either a

predominantly northeastern or southern genotype (Figures 2A, 2B). Although population-mean admixture was similar to Iberia (Figures 2A, 2B), the population-level variance in admixture proportions in Greece and the Aegean were significantly higher than in Iberia (non-parametric bootstrap test, $p<10^{-6}$). The same result was found using the hybrid index (HI) (Buerkle 2005) instead of admixture proportions (non-parametric bootstrap test, $p<10^{-6}$). This result indicates limited admixture between individuals from northeastern and southern origin within the secondary contact zone.

Locally adapted clinal color variation

Plumage coloration is sexually dimorphic with a bimodal distribution in both sexes (Figure 4A), and showed a pronounced geographic structure. Barn owls in the south are white, while in the north they get gradually darker rufous towards the east (latitude: t=5.44, $p<10^{-4}$; longitude: t=-7.60, $p<10^{-7}$; interaction: t=8.02, $p<10^{-7}$; R^2=0.89) (Figure 4B). Ring distance from the Middle East explained 22% of color variation (t=2.72, p=0.011), and only the Middle East, Crete, Greece, and the Aegean did not follow a stark spatial pattern (Figure S9). With these populations excluded, ring distance alone explained 85% of color variation (t=11.23, $p<10^{-9}$), which was more than explained by flight (t=-3.76, p=0.001, R^2=0.39) or overland distance (t=-5.28, $p<10^{-5}$, R^2=0.56).

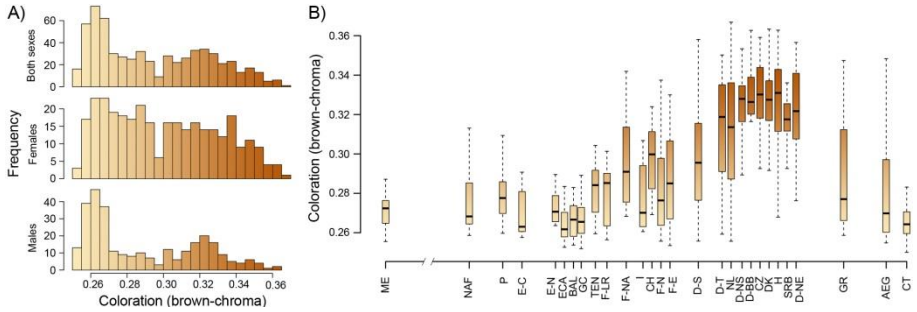

Figure 4. Color distributions in barn owls. **A)** Color frequency distributions for both sexes separately and jointly. **B)** Geographic distribution of coloration. Boxes are provided at approximate ring distance from the Middle East (exact distances overlap between some populations).

Color differentiation among populations was marked, and followed an isolation-by-distance pattern best explained by spatial proximity (flight distance) of populations (Table 1). F_{ST}-P_{ST} comparisons between mainland populations showed that color differentiation (overall P_{ST}=0.40) exceeds predictions from neutral genetic markers (overall microsatellite F_{ST}=0.045) ten times (Figure 5). Concordantly, P_{ST} differed significantly from the expected distribution of neutral differentiation (1973) ($p<10^{-15}$), demonstrating that genetic drift alone cannot explain color differentiation. Bayesian quantitative genetic modelling (Ovaskainen et al. 2011) also rejected neutral evolution as the only driver of color differentiation (S>0.999). These results provide evidence for diversifying selection on coloration at the scale of the Palearctic distribution of barn owls and confirm previous results found at a smaller scale (Antoniazza et al. 2010).

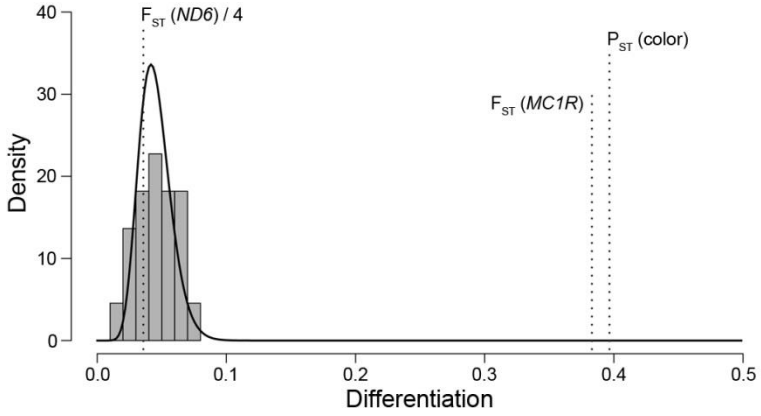

Figure 5. F_{ST}-P_{ST} comparisons. Overall differentiation in color (P_{ST}) and at *MC1R* are compared against neutral genetic differentiation at nuclear microsatellite markers (grey bars) and at the mitochondrial *ND6* locus. Grey bars: histogram of F_{ST} of the 22 nuclear makers. Solid line: theoretical F_{ST}-distribution (Lewontin & Krakauer 1973). Broken lines: Overall differentiation at *ND6* corrected for differences in nuclear and mitochondrial effective population sizes, and differentiation in color and at *MC1R*.

Genetic basis and origin of coloration

Sequencing of the melanocortin-1-receptor (*MC1R*) gene in 671 individuals revealed a frequent non-synonymous Ile-Val polymorphism at amino acid position 126 (Text S3). Heterozygotes and Ile-homozygotes were significantly more rufous than Val-homozygotes (Figure 6). *MC1R* genotype, sex, and population structure together explained 63% of color variance (sex: t=-9.31, $p<10^{-15}$; F_{ST}: t=3.14, p=0.002; *MC1R* (Val-Ile): t=-4.65, $p<10^{-5}$; *MC1R* (Val-Val): t=-24.35, $p<10^{-15}$). Analyses by sex and population confirmed this result (Figure S10, Text S4). Given their strong correlation with coloration, we hereafter refer to the Ile and Val variants as $MC1R_{RUFOUS}$ and $MC1R_{WHITE}$, respectively.

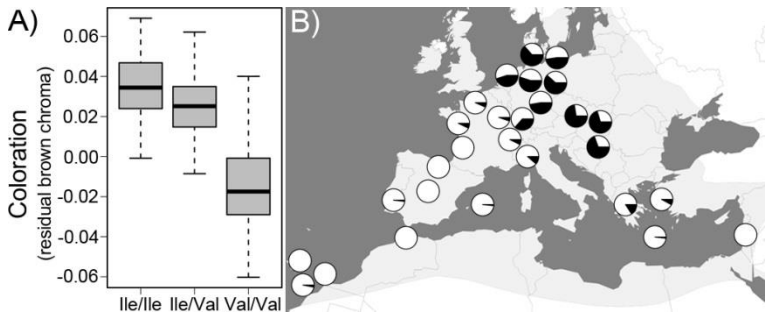

Figure 6. A) Relationship of coloration with *MC1R* genotype in barn owls. Values provided for coloration are residuals after correcting brown chroma for population structure and sexual dimorphism. **B)** Spatial frequency distribution of the $MC1R_{WHITE}$ (white) and $MC1R_{RUFOUS}$ variants (black).

MC1R allele frequencies showed a pronounced geographic structure (overall F_{ST}=0.383, range 0-0.797, Figures 5, 6B, S2B), and population structure (F_{ST}) at *MC1R* was strongly correlated with color differentiation (P_{ST}) (Mantel R=0.823, p=0.001), but much less with neutral genetic differentiation or spatial distances (max 31%, Table 1). Partial Mantel tests demonstrated that neither geography nor neutral genetic differentiation explain color differentiation nearly as well as *MC1R* (Table 1). Together with the significantly stronger differentiation than predicted from neutral genetic differentiation (Figure 5), this suggests that neutral evolution alone cannot explain population structure of coloration and of the underlying *MC1R* genotypes. The stronger correlation of geographic structure of coloration (and *MC1R*) with spatial proximity of populations (i.e. flight distance), rather than with ring distance (Table 1) suggests that selection mediated by locally prevailing environmental factors rather than colonization history determines the geographic structure observed for coloration and *MC1R*, and provides additional evidence for the local adaptation of coloration.

In order to evaluate the relative ages of the non-synonymous *MC1R* variants, we analyzed linked genetic variation. Ten low-frequency polymorphisms linked to $MC1R_{WHITE}$ (0.2-3.1% within $MC1R_{WHITE}$) were identified from full-length coding sequences (N=870

alleles). Conversely, only two polymorphisms were found linked to *MC1R*$_{RUFOUS}$ (N=252 alleles), both occurring at minimal frequency within *MC1R*$_{RUFOUS}$ (1 and 2 copies, respectively). Their higher frequencies on *MC1R*$_{WHITE}$ indicate that they originally evolved on this allele and got linked to *MC1R*$_{RUFOUS}$ by recombination. Non-parametric bootstrap tests show that the over ten times lower polymorphism linked to *MC1R*$_{RUFOUS}$ ($\pi=2.16 \cdot 10^{-6}$) than to *MC1R*$_{WHITE}$ ($\pi=2.66 \cdot 10^{-5}$) is not an artifact from unequal sample sizes ($p<10^{-6}$). The significantly elevated variation linked to *MC1R*$_{WHITE}$ together with sequence information from five outgroup individuals establish *MC1R*$_{WHITE}$ as the ancestral variant.

Our results therefore demonstrate that a derived non-synonymous mutation at *MC1R* was involved in the evolution of a novel rufous barn owl phenotype, which is locally adapted to environmental conditions prevailing in Northeastern Europe. The lack of private variation linked to the derived *MC1R*$_{RUFOUS}$ variant together with this variant's geographic distribution suggest, that it evolved early during the colonization of Europe.

Linking coloration and genetic ancestry

If the rufous phenotype evolved early during the colonization of Europe and is locally adapted, selection may be expected to keep it restricted to a European genetic background. Indeed, even after taking into account sexual dimorphism, *MC1R* genotype, and population structure, the genetic ancestry of individuals estimated in Bayesian analyses of population structure (Q) explained a significant amount of color variation (sex: $t=-10.08$, $p<10^{-15}$; *MC1R*(Val-Ile): $t=-4.73$, $p<10^{-5}$; *MC1R*(Val-Val): $t=-21.48$, $p<10^{-15}$; FST: $t=2.21$, $p=0.027$; Q: $t=7.65$, $p<10^{-13}$; $R^2=0.67$). Still, this result may establish by the spatial correlation of coloration with genetic structure, and potentially insufficient correction for genetic structure

in our model. However, especially in the secondary contact zone rufous and white phenotypes are expected to be associated with predominantly northern and southern genetic ancestry, respectively, if gene flow between the terminal populations within the ring is restricted. This prediction is supported by the observation that Greece is not only where individuals of northeastern and southern ancestry meet, but is also the region with highest variance in plumage coloration (Figure 4B).

We therefore tested whether in the secondary contact zone in Greece and the Aegean rufous coloration was associated with northeastern ancestry by estimating genetic ancestry using (i) admixture proportions (Q), (ii) the first axis of an individual-based principle component analysis (PCA), and (iii) the hybrid index. All analyses confirmed that in the secondary contact zone darker rufous individuals have elevated northeastern ancestry (Figure S11; Q: sex: t=-2.95, p=0.006; *MC1R*(Val-Ile): t=-0.60, p=0.55; *MC1R*(Val-Val): t=-2.36, p=0.025; ancestry: t=2.53, p=0.017, R^2=0.60; PCA, sex: t=-2.45, p=0.021, *MC1R*(Val-Ile): t=-0.74, p=0.464; *MC1R*(Val-Val): t=-2.49, p=0.019, ancestry: t=2.35, p=0.026, R^2=0.59; for HI, sex: t=-2.53, p=0.017, *MC1R*(Val-Ile): t=-0.86, p=0.397; *MC1R*(Val-Val): t=-2.63, p=0.013, HI: t=-3.15, p=0.004, R^2=0.64). No other population showed the same consistent correlation between color and genetic ancestry. The secondary contact zone in Greece and the Aegean is thus the only region with evidence for linkage disequilibrium of coloration with neutral genetic ancestry.

Discussion

The present study establishes the barn owl as a ring species around the Mediterranean, and shows how local adaptation during range expansion may lead to the

evolution of reproductive isolation in the face of gene flow. We first present the evidence for incipient ring speciation in European barn owls and then discuss our results in the light of the origins of adaptive genetic variation. Finally, we consider how our results may elucidate how range expansion dynamics and local adaptation can contribute to the build-up of reproductive isolation.

Speciation in a ring around the Mediterranean

With a high level of genetic diversity and allele frequencies closest to outgroups, the Middle East represents the most likely origin of the circum-mediterranean colonization, which first reached North Africa and Iberia. From this refugial area barn owls subsequently colonized the European continent northeastward in a range expansion during which genetic diversity diminished successively. The closest relationship of the populations in the Middle East and North Africa/Iberia, and the strong unidirectional expansion signal in diversity exclude alternative colonization scenarios invoking multiple refugia (Text S2). The steep diversity gradient and shallow population structure throughout Europe suggest that the expansion into Europe happened quickly and recently. Recent spatially-explicit analyses of demographic history confirm this result, dating the onset of colonization to after the last glacial maximum ~20,000 years ago (Antoniazza et al.). In the east, a second, smaller colonization closed the ring around the Mediterranean, having advanced over the Bosporus into Southeastern Europe (Greece) (Figure 3). Despite the vast geographic distance covered by the ring (>8,000 km), differentiation along the ring is shallow (nuclear F_{ST}: mean mainland=0.027, max=0.085), suggesting that populations are interconnected by high rates of ongoing gene flow. Model-based estimates of gene flow and field observations show that

in Europe almost 40% of barn owls disperse 50 km or further after fledging (Antoniazza et al. submitted). Gene flow appears thus more restricted than across the willow warbler ring (Bensch et al. 2009), which covers around 4,000 km. Compared to greenish warblers, which show deep phylogeographic breaks in mitochondrial variation (Irwin et al. 2001b), barn owls have way lower mitrochondrial diversity and display only gradual shifts in mitochondrial haplotype frequencies. The barn owl system thus appears to be younger than the greenish warbler ring, and despite the larger geographic scale of the barn owl ring, barn owl populations appear more interconnected by gene flow (greenish warbler ring: 6-7,000 km around the Tibetan Plateau).

The secondary contact zone in Greece is characterized by unique distributions of genotypes, genetic diversity, and color phenotypes reminiscent of hybrid zones. Admixture analyses indicate that these patterns are a result of limited introgression at secondary contact. This conclusion is supported by several other lines of evidence. First, in Crete despite historical introgression at mitochondria and evidence for contemporary immigration from the north, introgression is strongly limited for color and the nuclear genome. Likewise, no nuclear introgression from the south is detected in Eastern European populations. Second, with free introgression among terminal taxa, linkage disequilibrium between color phenotype and genetic ancestry would be expected to break down after secondary contact, unless maintained by strong persistent gene flow from the north and south. It appears unlikely that the secondary contact zone should receive more such immigrants than the genetic transition zone in Iberia. The significant association of rufous coloration with northeastern genotypes in Greece thus provides additional support for limited introgression among terminal taxa.

Our results demonstrate that following the last glaciation, settling out from the Middle East barn owls colonized North Africa and Europe in a ring around the Mediterranean, and met in secondary contact in Southeastern Europe, where they appear to be at the verge of speciation.

Adaptive evolution of coloration from novel genetic variation

An important question in evolutionary biology addresses the origin of adaptive genetic variation: does local adaptation evolve from genetic variation segregating in populations (standing variation), or from novel, derived variants (Barrett & Schluter 2008)? Studies demonstrating local adaptation of color phenotypes and identifying the underlying genes have previously contributed remarkable insights in this respect (Hoekstra et al. 2006). The young age of the color cline in the barn owl that accompanies the gradual genetic changes within Europe might have suggested that color adaptation in the barn owl evolved from genetic variation present at the onset of colonization. However, as we discuss, the colonization of continental Europe indeed may only have been enabled by the evolution of a novel genetic variant.

Local adaptation of barn owl coloration is highlighted by one of the most remarkable findings of the present study: Differentiation in color and the underlying gene (*MC1R*) is strongest within the geographic region with the by far weakest – indeed almost absent – population structure (Central, Northern, and Eastern Europe). Together with quantitative genetic analyses and previous ABC-analyses (Antoniazza et al. submitted) this demonstrates local adaptation of coloration and/or phenotypic traits linked by pleiotropy (Ducrest et al. 2008). In accordance with this result, previous studies highlight differences in the ecology of

alternative barn owl color phenotypes: Rufous owls exploit more open habitat, and accordingly feed predominantly on voles rather than muroids both in Switzerland and Israel (Charter et al. 2012; Roulin 2004). Females choose breeding habitat to match their color phenotype, with rufous females producing more offspring in open habitat (Dreiss et al. 2012). Rufous males make higher reproductive investments and produce more fledglings (Roulin et al. 2001). Rufous juveniles grow better under harsh conditions when food is limited (Roulin et al. 2008) and show more altruistic behavior (Roulin et al. 2012). The multidimensional ecological differences between barn owl color phenotypes appear to contribute to local adaptation even at a very local scale (Dreiss et al. 2012), and are situated in the range of ecological differentiation typically found within young adaptive radiations such as in cichlid (Seehausen 2006) and stickleback fish (McKinnon & Rundle 2002), but for which only few examples exist in birds (Edelaar et al. 2012; Ryan et al. 2007; Schluter et al. 1985). Taken together, the currently available evidence unanimously suggests that the ecology of rufous individuals is better adapted to the open and harsh environment found in continental Europe, and opens a scope for ecology-driven selection to result in isolation-by-ecology and potentially ecological speciation within European barn owls, evidence for which is rare in birds (Price 2007).

Previous work on color-related adaptation identified *MC1R* as a major genetic determinant of color polymorphism in a wide range of species (Hoekstra et al. 2006; Mundy et al. 2004; Theron et al. 2001; Våge et al. 1997). The identification of this gene as major contributor to color variation in barn owls greatly helps understanding the evolutionary history of color adaptation. The $MC1R_{RUFOUS}$ variant predominant in the most recently colonized northeast of Europe appears to be both derived and way younger than the ancestral $MC1R_{WHITE}$ variant. The young age of this novel variant implies a recent origin of

the rufous barn owl phenotype. Together with its almost complete absence in the south of the range this suggests that the *MC1R*$_{RUFOUS}$ variant arose not before the colonization of Central Europe. Interestingly, several studies link rufous coloration of barn owls to increased juvenile dispersal distance (Roulin 2013; Van den Brink et al. 2012) and phenotypic traits enhancing long-distance flight (longer wings, Charter et al. 2012; Roulin 2004), and may suggest that rufous coloration is connected to a disperser phenotype. Theoretical models suggest that in such cases increased dispersal propensity itself could have conferred rufous birds with an additional advantage during range expansion (Travis & Dytham 2002).

It thus appears that the rapid colonization of Northern and Eastern Europe by barn owls may have been triggered by the novel phenotype's ability to cope with harsher environments, and accelerated by this phenotype's enhanced dispersal ability.

Range expansion, local adaptation, and the evolution of reproductive barriers

Range expansions leave remarkable traces in the genetic make-up of populations by influencing rates of fixation, the direction of introgression, and the relative strength at which selection can operate (Excoffier et al. 2009). Barn owls may represent a unique system to study the interplay of range expansion and local adaptation, and how in conjunction they may contribute to the formation of reproductive barriers.

By enabling barn owls to live in a harsher environment and disperse over longer distances, the evolution of a new phenotype may have resulted in a sudden burst of colonization of Central Europe after the last glaciation. For the evolution of reproductive barriers two aspects of this scenario appear particularly interesting. Both (i) pervasive selection on *MC1R* and (ii) rapid range expansion have the potential to accelerate rates of

evolution and lead to a coincidental accumulation of genetic incompatibilities. During adaptation polymorphisms physically linked to the positively selected variant can hitchhike along and increase in frequency, provided the net fitness change is positive (Hill & Robertson 1966; Maynard Smith & Haigh 1974). With strong selection on the novel MC1R variant, even previously slightly counter-selected (nearly neutral) linked polymorphisms may thus have increased in frequency. Likewise, allelic turnovers are accelerated during range expansion due to enhanced rates of genetic drift, and disadvantageous allele combination can persist at the colonization front owing to the decreased efficiency of selection (Burton & Travis 2008; Peischl et al. 2013). As we demonstrated, coloration and *MC1R* have evolved under local adaptation in barn owls and not as a byproduct of expansion dynamics (Antoniazza et al. submitted). In contrast, for genes less constrained by selection especially the rapid colonization of Europe may have provided ample opportunity to overcome fitness disadvantages of previously counter-selected allelic combinations and shift genetic variants towards new coadapted combinations which contribute to reproductive isolation at secondary contact (Mayr 1963). Although gene flow between the ancestral white and derived rufous phenotype appear restricted in secondary contact, and a direct involvement of coloration in reproductive isolation has been demonstrated in various species (e.g. Kronforst et al. 2006; Seehausen et al. 2008), in barn owls it appears more likely that selection is targeted also towards life history traits linked to coloration via the melanocortin system, rather than coloration alone (Ducrest et al. 2008). Simultaneous selection on both coloration and other phenotypes linked to the melanocortin system may further have accelerated the evolution of the rufous phenotype and indirectly contributed to the evolution of reproductive barriers.

Conclusions & Prospects

We showed that European barn owls form a ring species around the Mediterranean Sea, and demonstrate that the locally adapted color cline found within the ring evolved by the adaptive evolution of a novel *MC1R* variant. We propose that the evolution of the novel rufous phenotype is associated with the persistence of barn owls in the harsher Northeastern European environmental conditions, and that the colonization of Europe was additionally accelerated by enhanced dispersal abilities of the novel color phenotype. Enhanced rates of evolution during the colonization may have contributed to the accumulation of intrinsic reproductive barriers that limit introgression at secondary contact. Future studies based on genome-wide data will have to provide conclusive evidence for reproductive isolation of the terminal forms of the ring, and will allow identifying genomic regions resistant to introgression, and trace their evolution by population genetic studies along the barn owl ring species.

Summary Experimental Procedures

724 barn owls were sampled in 28 localities all around the Mediterranean and throughout Europe. Based on data from 22 microsatellite loci and 411 bp of the mitochondrial ND6 gene (Table S1) neutral genetic population structure and colonization history were inferred using principle coordinate analyses, Mantel tests, and Bayesian admixture analyses in STRUCTURE (Pritchard et al. 2000). Individual coloration was measured in 626 individuals using photospectometry. 543-998 bp of the *MC1R* gene were sequenced for 671 individuals (Table S1). A link between coloration and variation at *MC1R* was investigated using linear models correcting for sexual dimorphism and geographic

structure. Linked polymorphism levels and sequences from outgroups from California, USA (T. a. pratincola, N=65), Australia (T. a. delicatula, N=19), and Singapore (T. a. javanica, N=11) were used to infer the ancestral and derived states of the *MC1R* polymorphism linked to coloration. F_{ST}-Q_{ST} analyses and a Bayesian quantitative genetic model (Karhunen et al. 2013; Ovaskainen et al. 2011) were used to infer the role of local adaptation in maintaining color differentiation. Patterns of introgression in the secondary contact zone were studied using an array of admixture analyses (Buerkle 2005; Pritchard et al. 2000) combined with linear models linking individual neutral genetic ancestry with coloration.

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Supplemental information

Supplemental Experimental Procedures

Sampling, molecular analyses, and genotyping

A total of 724 unrelated barn owls have been sampled in 28 different localities around the Mediterranean Sea and all over the European continent (Figure 1). Genomic DNA from all individuals was extracted from the basal 1 millimetre of breast feather quills, from blood, or from muscles stored in 96% ethanol. DNA extractions were performed on a BioSprint 96 extraction robot using the BioSprint 96 DNA blood kit, or with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The sex of most individuals was determined using the molecular method described in Py et al. (2006) (sexing did not work for 10, 2, and 2 samples from museum skins from North Africa, Italy, and Greece, respectively, and for 4 individuals from the Balkans and 1 from the Eastern Canaries). This method distinguishes sexes based on a length-dimorphism of the *SPINDLIN* gene between the Z and W chromosomes.

All 724 individuals were genotyped for 22 microsatellite markers that show no evidence for null alleles and no constant deviation from Hardy-Weinberg genotype proportions (Table S2). Polymerase chain reactions (PCR) were performed in five PCR multiplexes (Table S2) using the Multiplex PCR Kit (Qiagen). Reactions were carried out in a final volume of 8 µl, and contained 2.5 µl mix MM (Qiagen), primers as indicated in Table S2, and 12 ng of DNA. PCR conditions included an initial step of denaturation for 15 min at 95 °C, 34 cycles of 30 sec denaturation at 94 °C, annealing for 1 min 30 sec at 57 °C, and elongation at 72 °C for 1 min. Final elongation for 30 min was conducted at 60 °C. Fragment analyses were run on an ABI 3100 sequencer with a ROX 500 size standard and allele lengths were assigned using Genemapper 4.0 (Applied Biosystems, Zug, Switzerland).

411 bp of the mitochondrial NADH-dehydrogenase-6 (*ND6*) locus were sequenced for 424 individuals (on average 16 per population, Table S1) using primers L14906-TyalND6 (5'-CGA GAT AGC CCA CGA ACA AGC-3') and H15378-TyalND6 (5'-GAG GTG CGA GTC TGG TTT TGG-3'). Reactions were carried out in a final volume of 25 µl, containing 1x Buffer Gold, 2 mM MgCl2, 1x Q-Solution (Qiagen), 0.2 mM each dNTP, 0.5 µM each primer, 1 unit Taq Gold (Applied Biosystems), and 20 ng DNA. PCR conditions included an initial step of denaturation for 7 min at 95 °C, 35 cycles of 30 sec at 95 °C, 45 sec at 62 °C, 45 sec at 72 °C, and a final elongation for 7 min at 72 °C.

The melanocortin-1-receptor (*MC1R*) gene was repeatedly shown to underlie melanin-based coloration in birds and mammals (Dessinioti et al. 2011; Roulin & Ducrest 2013). In order to study whether this gene explains coloration in barn owls, 543 bp (N=211) or the whole 998 bp (N=417) of the exon were sequenced for 671 individuals (Table S1) using primers MC1R_34Fw (5'-GGG ACC CCG GGG TTG AGG CG-3') and MC1R_568Rev (5'-GGC AGA GGA GGA TGG CGT TGT TGC G-3') for the short and MC1R_34Fw (5'-GGG ACC CCG GGG TTG AGG CG-3') and MC1R_969Rev (5'-GCG TTA ACC CGC GTC CCG CTG C-3') for the long fragment with the following PCR conditions: 95 °C for 3 min followed by 34 cycles at 94°C for 40 sec, 68°C for 40 sec, 72°C for 60 sec and then final extension at 72°C for 10 min with 250 nM of the above primer pairs, 1x Qiagen buffer, 2.5 mM MgCl2, 0.2mM dNTPs, 1x Q solution (Qiagen), 1 U Taq DNA Polymerase (Qiagen), and 50 ng DNA in 50 µl. The amplified DNA fragments were then purified using the MinElute PCR purification kit (Qiagen). Sequencing was conducted at Microsynth (Balgach, Switzerland) and at the university facility on a 3130XL Genetic Analizer (Applied Biosystems) with a special sequencing protocol in a final volume of 10 µl containing 2 µl of Big Dye v3.1, 2 µl of 5x Q solution (Qiagen), 1 µl of 10 µM of one of the primers and 2 µl of amplified DNA with

amplification at 98°C for 2 min, 39 cycles at 96°C for 15 sec, 60°C for 15 sec, 60°C for 3 min. For museum samples and a few low-quality DNA samples amplification of these fragments was not possible (N=43), and we used an allelic discrimination (AD) assay for the Val126Ile mutation. For this we pre-amplified a shorter fragment of MC1R spanning 255 bp with primers MC1R_198fw (5'-CCT GCA CTC GCC CAC GTA CTA CTT C-3') and MC1R_453Rev (5'-GTG GTA GCG CAG GGC GTA GAA GAT-3') followed by a nested PCR of 91 bp with primers MC1R_V126I_fw (5'-CAT GGA CAA CGT CAT CGA-3') and MC1R_V126I_rev (5'-GCG TAG AAG ATG GTG ATG TA-3') and fluorescence marked specific probes V126I_wt_Fam_B1 (FAM-5'-TGC AGC TCC GTC GTG TCC TC-3'-BHQ1), and V126I_mut_AT550_B2 (ATTO-550-5'-TGC AGC TCC ATC GTG TCC TC-3'-BHQ2) for the wild type and mutant alleles, respectively. Pre-amplification PCRs were performed in 20 µl using exactly 20 ng DNA, 250 nM of primers MC1R_198Fw and MC1R_453Rev, 2.5 mM MgCl2, 0.2 mM dNTPs, 1x Q solution (Qiagen), and 0.2 U of Taq Qiagen with the following cycle: 95 °C for 5 min, 34 cycles at 94 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 30 sec. The quantities of pre-amplified PCR products were compared using a 2% agarose gel, adjusted among each other, and diluted 100x before the AD assay. 24 µl of AD assay were run in an ABI 7500 qPCR machine (Applied Biosystems) using 2 µl of diluted DNA with 12 µl of qPCR MasterMix Plus Low ROX w/o UNG 2x (Eurogentec, Liege, Belgium) and 300 nM of the primers MC1R_V126I_fw and MC1R_V126I_rev, 100 nM of V126I_wt_Fam_B1, and 250 nM of V126I_mut_AT550_B2 with the ABI 7500 recommended cycling conditions except for an annealing and extension temperature of 57°C. Each qPCR plate contained three positive samples (corresponding to both homozygous and the heterozygous genotypes) and at least two negative controls. The plates were then read with the allelic discrimination program of the ABI 7500 machine.

Microsatellite markers were genotyped in Gene Mapper version 4.0. Sequences (*ND6* and *MC1R*), assembled and edited in CodonCode Aligner version 3.7.1, and aligned using the Clustal W algorithm (Thompson et al. 1994) in BioEdit (Hall 1999). Polymorphisms in the MC1R sequences were statistically phased using the PHASE software (Stephens et al. 2001) implemented in DnaSP 5.10.01 (Librado & Rozas 2009) using 1000 burnin and 1000 post-burnin iterations and a thinning interval of 10.

Neutral genetic population structure and admixture analyses

In order to infer neutral population structure and diversity, pairwise fixation indices (F_{ST}) between populations and allelic richness (AR) based on microsatellite and mitrochondrial data were estimated in R using the hierfstat v4-10 package (Goudet 2005). The same package was used to perform Principle Coordinate Analyses (PCoA) based on pairwise F_{STs}. To complement PCoA, Mantel tests were performed in R using the ecodist 1.2.7 package (Goslee & Urban 2007). A haplotype network for *ND6* was constructed using TCS 1.21 (Clement et al. 2000).

Linear models were used to test whether population genetic structure (as estimated by PCo axes) and genetic diversity (AR) are related to geographic proximity measured as flight and shortest overland distance, or to ring distance (see Figure S5 for an example). Flight distance and the shortest overland distance were used, because on one hand barn owls have good flying abilities and colonized many far off-shore islands, but on the other hand may still avoid flying over water. Ring distance was modeled as the distance between populations within a clock-wise ring starting in the Middle East and ending in Crete. The start and end point were determined from the population structure depicted by PCoA (Figures 1B, S3). All distances were measured manually in Google Earth. Ring distance was measured as

the shortest overland distance from the Middle Eastern population clock-wise in a ring around the Mediterranean.

In order to estimate which populations/clusters exhibit allele frequencies closest to outgroups, we ran two analyses. First, we ran STRUCTURE 2.3.4 (Pritchard et al. 2000) (with a burnin of $5{\cdot}10^4$ and $5{\cdot}10^5$ post-burnin generations) including outgroup populations from California, United States (T. a. pratincola, N=65), Australia (T. a. delicatula, N=19), and Singapore (T. a. javanica, N=11), and estimated neighbor-joining (NJ) trees based on the allele-frequency divergence (net nucleotide distance) among clusters to infer the relationships between the inferred clusters. We ran 10 iterations for each number of clusters (K), and increased K until no new cluster splitting off one or several populations was observed (at K=7). When more than one pattern of clustering was observed at a given K the one with higher likelihood and lower variation in likelihood was selected. Second, we estimated a population tree using F_{ST}. Three microsatellite markers showed more than 10% missing data in these populations, and were excluded from both analyses (Tak.Oeo53, Ta.305, Ta.408). As island populations showed increased differentiation relative to the other populations, and to avoid problems connected with long-branch attraction of the NJ method, island populations were excluded from both analyses.

Bayesian clustering analyses implemented in STRUCTURE were performed using microsatellite data in order to detect the number of genetic clusters, and estimate admixture proportions for each individual. K from K=1 to K=10 were tested. Analyses were run for all populations and for mainland populations plus the Aegean exclusively. The latter was included with mainland populations because it was a focal population for admixture analyses and because of the Aegean islands' proximity to the mainland and their genetic diversity very similar to the neighboring mainland population in Greece (the other island

populations show much lower genetic diversity than close-by mainland populations). Ten runs each with a burnin of 10^5 and 10^6 post-burnin generations were performed for each K using an admixture model with correlated allele frequencies. The number of clusters was inferred using the ΔK-method (Evanno et al. 2005) implemented in STRUCTURE HARVESTER (Earl & Vonholdt 2012).

To test whether the mean and variance in admixture proportions observed in the secondary contact zone in Greece and the Aegean differed significantly from the ones in other populations with similar admixture proportions (Portugal, Central and Northern Spain), we performed non-parametric bootstrap tests. As no significant differentiation of the populations in the secondary contact zone (Greece, Aegean) was observed, these populations were combined for the following analysis. Random samples the same size of the combined Greek and Aegean sample (N=46) were drawn from the admixture proportion (Q) distributions of the combined Iberian populations (N=61, these populations show the most similar admixture patterns to Greece). The same procedure was performed using the hybrid index (HI) (Buerkle 2005) instead of Q. For each analysis 106 random samples were drawn with replacement to generate expected distribution of the variance in admixture proportions. HI was estimated using the "introgress" package (Gompert & Alex Buerkle 2010) in R. To estimate HI, "southern" and "northeastern" parental populations were defined from STRUCTURE results, by using individuals with a respective $Q \geqq 0.97$ and without missing data that from Northern and Eastern Europe (northeastern lineage), and from the Middle East, Crete, and the Canary Islands (southern lineage), respectively. The northeastern parental population thus consisted of 78 individuals from the Netherlands (n=10), Niedersachsen (n=15), Brandenburg (n=8), Denmark (n=14), Northeastern Germany (n=8), Hungary (n=11), Czech Republic (n=4) and the Balkans (n=8). The "southern" parental

population consisted of 24 individuals from the Middle East (n=5), Eastern Canaries (n=6), and Crete (n=13).

Measurement and quantitative genetics of color phenotypes

For 626 individuals breast feathers were available and measured for coloration. Pheomelanin-based plumage color of each individual bird was measured from one to five breast feathers (mean: 4.15, SD: 1.08, depending on the number of feathers available). To measure color, reflectance spectra from four points per breast feather were captured with a USB4000 spectrophotometer (Ocean Optics, Dunedin, FL, USA) and a DH-2000-bal dual deuterium/halogen light source (Mikropackan, Mikropack, Ostfildern, Germany). For each reflectance spectrum, the brown chroma was calculated following Montgomerie (2006). The brown chroma represents the contribution of the red part of the spectrum (600–700 nm) to the complete visible spectrum (300–700 nm). For each individual, the brown chroma was averaged per feather (average among point measurements) and then per individual (average among feathers). The repeatability of assessing coloration was very high (97.6% of among individual variance) as shown by the repeated measurement of coloration of 14 individuals twice one year apart.

To test whether the color differentiation among populations evolved by genetic drift exclusively or whether local adaptation played a role in its evolution, we used two complementary, recently proposed approaches. The first is derived from classic F_{ST}-Q_{ST} comparisons (Brommer 2011; Leinonen et al. 2013; Leinonen et al. 2008; McKay & Latta 2002; Merilä & Crnokrak 2001). This approach by Whitlock (Whitlock 2008), instead of directly comparing overall values of Q_{ST} and F_{ST} compares Q_{ST} estimates to the distribution of F_{ST}. For this, we estimated F_{ST} as outlined above, and obtained the theoretical distribution

from Lewontin and Krakauer (1973). P_{ST} (an analog of Q_{ST} based on phenotypic traits) for color differentiation was estimated using ANOVA with color as response variable and the populations of origin and sex as explanatory variables following Antoniazza et al. (Antoniazza et al. 2010). Variance components where then combined with the estimated heritability of coloration (Roulin & Dijkstra 2003). Following the general approach proposed by Whitlock (Whitlock 2008), Whitlock & Guillaume (Whitlock & Guillaume 2009) have proposed a parametric bootstrap approach to compare the overall Q_{ST} and F_{ST}. This approach was not applicable to our system because it implies a resampling of the variance components of Q_{ST} extracted from a quantitative genetics breeding design. As our quantitative measurements are field-based, the required variance components are not available in our case. We thus used the general idea of Whitlock's (Whitlock 2008) proposition and directly compared our empiric P_{ST}-estimates to the theoretical chi-squared distribution of Lewontin & Krakauer (1973) to obtain a p-value for the F_{ST}-Q_{ST} comparison. F_{ST} estimates for *ND6* (divided by four to account for the difference of effective population size of mitochondrial markers) and for *MC1R* were added for comparison. The second approach is a Bayesian quantitative genetic method suggested by Ovaskainen et al. (Ovaskainen et al. 2011). This method compares Bayesian estimates of population differentiation at neutral genetic makers and quantitative traits. To apply this method, we used the two recently published R packages RAFM (Karhunen & Ovaskainen 2012) and Driftsel (Karhunen et al. 2013). The AFM function of the RAFM package was used to estimate a co-ancestry matrix based on 22 microsatellites markers. This function uses a Bayesian implementation of the admixture F-model to infer pairwise population differentiation. Driftsel uses the color measurements along with the population of origin and sex, and compares their variation to the neutral genetic differentiation estimated from microsatellites. A neutrality test makes use of the S

parameter of the model to infer whether the magnitude and regime of selection, i.e. whether selection acted on the quantitative traits either towards different optima in different populations (S≈1), or towards the same selective optimum in all populations (S≈0).

Relating coloration to *MC1R* polymorphism, geography, and neutral genetic ancestry

To investigate whether *MC1R* polymorphism explains individual variation in pheomelanin-based coloration, linear models were estimated with coloration measured as brown chroma as response variable, and *MC1R* genotype at amino acid position 126 and sex as factors. Models were estimated for the entire data set, taking into account population structure by using the neutral genetic distance (F_{ST}) from the Middle East as covariate, and for each population separately. Population structure for the non-synonymous polymorphism at amino acid position 126 was estimated by calculating F_{ST} using hierfstat. We investigated how population structure at *MC1R* was correlated to color differentiation and spatial distances between populations using Mantel tests and partial Mantel tests as implemented in ecodist. To illustrate color variation within and between populations (Figure 4B), coloration was corrected for sexual dimorphism using a linear model.

In order to evaluate whether there were significantly more polymorphisms linked to the $MC1R_{WHITE}$ allele that was sampled more often, we applied a bootstrap randomization procedure on all variation observed across the full sequence (10 polymorphic sites, the polymorphism at amino acid position 126 was excluded). Only sequences covering all variable sites were used for this analysis (618 $MC1R_{WHITE}$, 252 $MC1R_{RUFOUS}$). Then we randomly sampled 252 out of the 618 full-length $MC1R_{WHITE}$ alleles for 106 times. Each time we estimated the mean number of pairwise differences (π) in the sample. The p-value of the

observed variation was then retrieved by comparing the observed π to the distribution obtained by bootstrapping.

Linear models were used to examine the hypothesis that within the secondary contact zone more rufous individuals have an elevated neutral genetic ancestry from Northeastern Europe. As the populations from Greece and the Aegean are not genetically differentiated, they were combined for this analysis. Models included coloration (brown chroma) as a response variable, and sex, *MC1R* genotype, and neutral genetic ancestry as explanatory variables. We used three approaches to estimate neutral genetic ancestry. The first models made use of the admixture proportions (Q) attributed to the "Northeastern" cluster by STRUCTURE for mainland populations (including the Aegean). Second, ancestry was approximated by individuals' first principal axis score of an individual-based Principal Component Analysis (PCA; PCA eigenvalue of first axis=5.7). The PCA was conducted in R using the adegenet 1.3-7 package (Jombart et al. 2008). Third, neutral genetic ancestry was expressed in terms of the hybrid index (HI) estimated as outlined above.

Supplemental texts

Text S1

The neighbor-joining (NJ) tree based on F_{ST} between mainland populations shows that the population from the Middle East is the most basal after the split of the outgroup populations. The next ones to split off are the ones from North Africa, Greece and Aegean, and Iberia (Figure S6A). The NJ trees estimated from allele-frequency divergence (net nucleotide distance) between clusters inferred by STRUCTURE confirm this result (Figures S6B-E). After the split of the outgroup populations from Europe (Figures S6B), the first

branching within Europe occurs at K=4 (Figure S6C) between clusters predominantly contributing to ancestry in (i) the Middle East, Iberia, and Italy, and (ii) the rest of Europe (populations in the secondary contact zone show a mixed composition). With the subsequent split of predominantly individuals from Hungary and the Balkans at K=5, the basal position of the cluster encompassing all individuals from the Middle East, and contributing to ancestry in Iberia and the secondary contact zone becomes apparent (Figure S6D). This basal position of the Middle Eastern population is reinforced at K=6, when individuals from Iberia and Italy split off into a cluster of their own (Figure S6E). No additional resolution could be reached at higher values of K.

Text S2

The geographic distribution of white and rufous barn owls lead Voous (1950) to propose that these barn owl phenotypes evolved in two refugia, in the Mediterranean and Southeastern Europe respectively. According to this hypothesis, the color cline between these regions would be a result of admixture after secondary contact. Together with previous evidence (Antoniazza et al. 2010) and spatially explicit ABC-modeling (Antoniazza et al. submitted) several lines of evidence clearly reject this hypothesis. The unidirectional decrease in genetic diversity (Figure 2D) is not expected with two disjunct refugia in Iberia and Southeastern Europe, respectively. In an alternative scenario with colonization of Europe from refugia in Iberia and the Middle East or on Crete, a close relationship of the latter two populations with the Southeastern European populations (Balkans, Hungary, Czech Republic) would be expected. Clearly, our results with a closer relationship of the populations in the Middle East and on Crete to populations on the Canary Islands and in Iberia (Figures 2A, S2) rule out this hypothesis.

Text S3

Sequencing of the melanocortin-1-receptor (*MC1R*) gene in 671 individuals revealed three non-synonymous polymorphisms, at amino acid positions 112 (Arg-His), 126 (Val-Ile), and 212 (Arg-Cys). The polymorphisms at positions 112 and 212 are rare and only occur in heterozygous state (two 112His alleles in Eastern France; one, 212Cys allele in Switzerland, one in Italy, two in Central Spain and eleven on the Baleares). Only the Ile-Val polymorphism at amino acid position 126 is frequent, with a total of 448 Val-homozygotes (66.8%), 110 Ile-homozygotes (16.8%), and 113 heterozygotes (16.4%).

Text S4

The relationship of coloration with *MC1R* genotype was confirmed also when data were analysed separately for each sex, and for each population. 53% and 72% of variance were explained for females and males, respectively, if analyses were carried out separately for each sex (females, F_{ST}: t=2.87, p=0.004; *MC1R*(Ile-Val): t=-2.10, p=0.036; *MC1R*(Val-Val): t=-13.68, $p<10^{-15}$; males, F_{ST}: t=1.20, p=0.230; *MC1R*(Ile-Val): t=-4.58, $p<10^{-5}$; *MC1R*(Val-Val): t=-21.97, $p<10^{-15}$). Apart from the populations in Portugal, Hungary, and the Balkans, in populations variable for *MC1R* the *MC1R* genotype had a significant (Gran Canaria, Baleares, Italy, Northern France, Eastern France, all German populations, Denmark, Netherlands, Czech Republic, Greece, Aegean, Crete) or nearly significant (France Nantes, p=0.056; Switzerland, p=0.053) effect on coloration (Figure S11), and a model including sex explained between 36% (Southern Germany) and 90% (Gran Canaria) of color variation in each population (mean 58% ± 16% sd).

Supplementary data

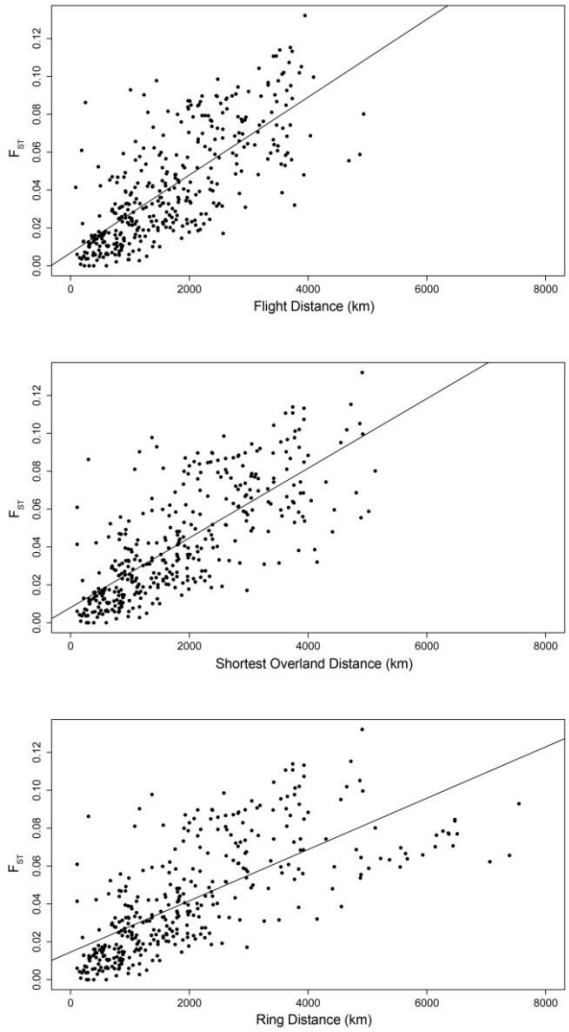

Figure S1. Isolation by distance patterns for different distance models in the barn owl. Regression lines are shown for illustrative purposes. Statistics for the Mantel regressions are provided in Table 1.

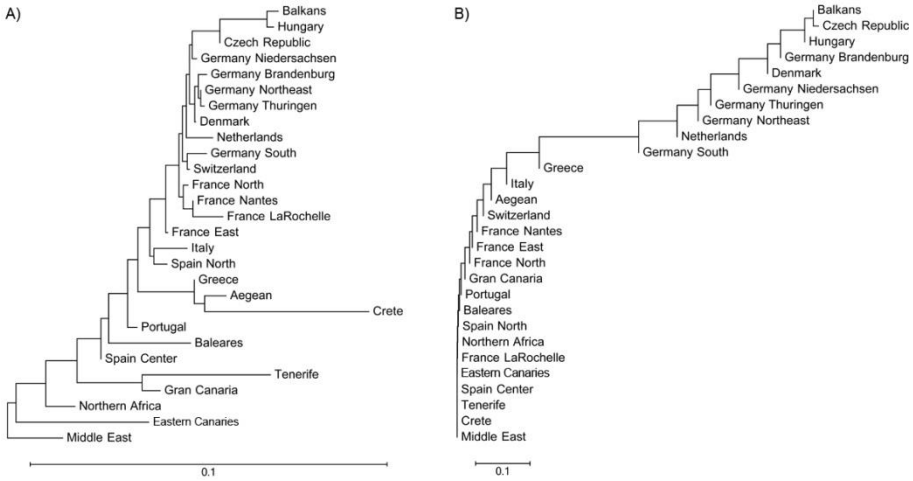

Figure S2. Neighbor-joining trees based on pairwise population differentiation (F_{ST}) estimated from **A)** 22 nuclear microsatellite markers and **B)** allele frequencies at *MC1R* amino acid position 126 in the barn owl.

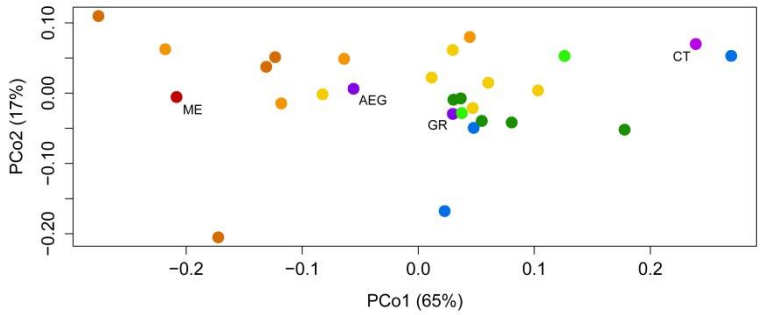

Figure S3. Principle coordinate analysis based on population differentiation (F_{ST}) at mitochondrial *ND6* in the barn owl. Labels and colors follow Figure 1.

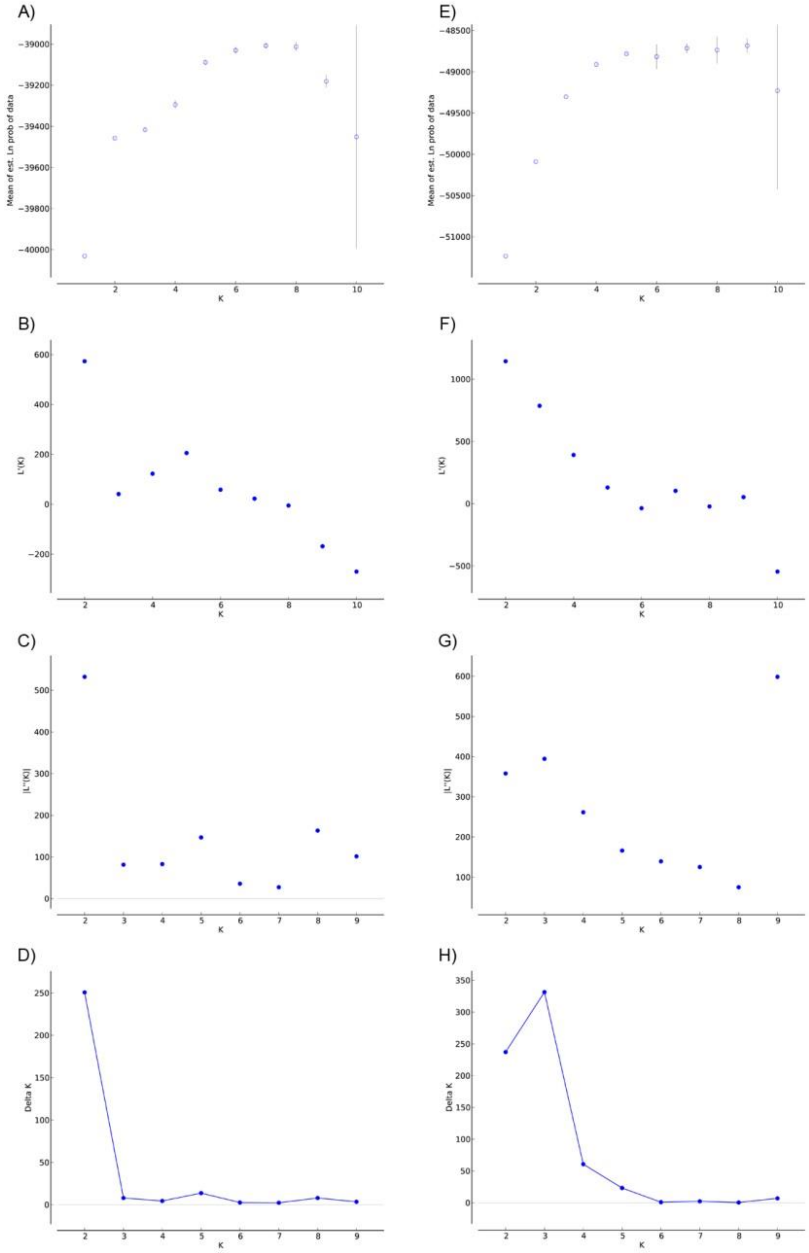

Figure S4. STRUCTURE results for mainland populations only (A-D) and for all populations (E-H). A,E) Lean likelihood ± standard deviation. B,F) Absolute change of the likelihood distribution (mean). C,G) Rate of change of the likelihood distribution (mean). D,H) Delta K.

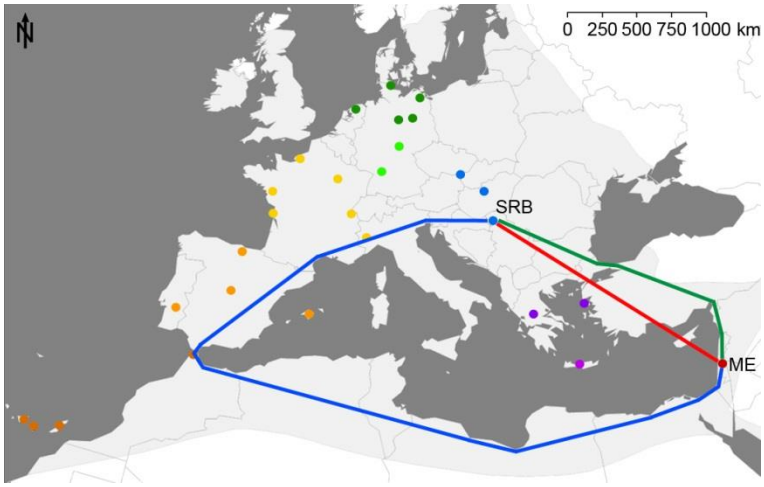

Figure S5. Alternative distance models. The example illustrates flight distance (red), shortest overland distance (green), and ring distance (blue) between the populations from the Middle East (ME) and the Balkans (SRB).

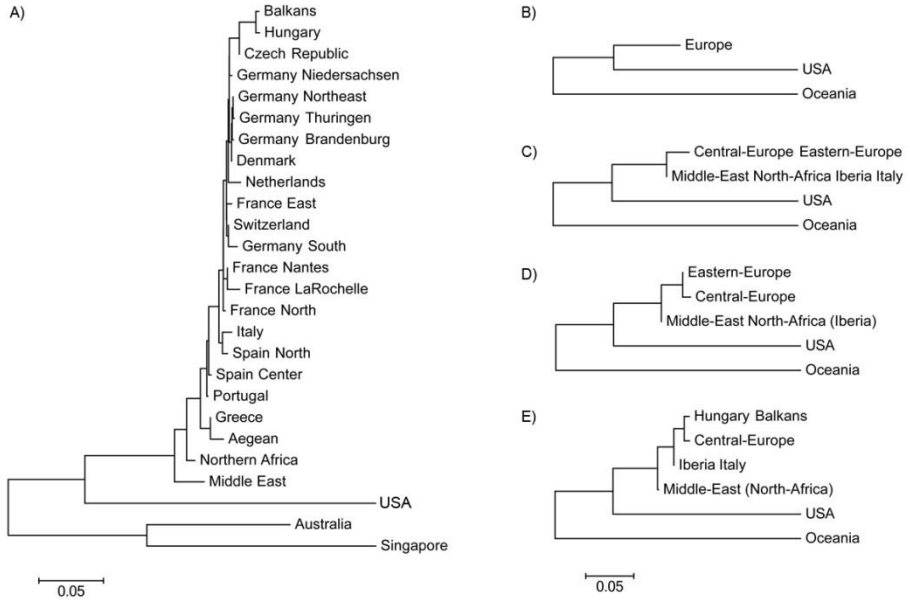

Figure S6. Population trees among European mainland populations rooted with outgroup populations from the United States and Oceania (Australia and Singapore). A) NJ-tree based on pairwise F_{ST} between populations. B) - E) NJ-trees based on on the allele-frequency divergence (net nucleotide distance) among clusters inferred by STRUCTURE for K=3 to K=6.

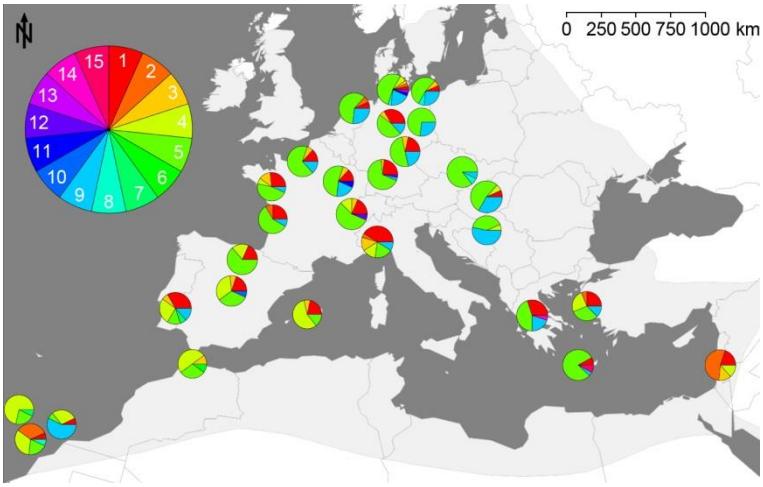

Figure S7. Spatial frequency distribution of mitochondrial ND6 haplotypes in the barn owl. The legend shows haplotype numbers.

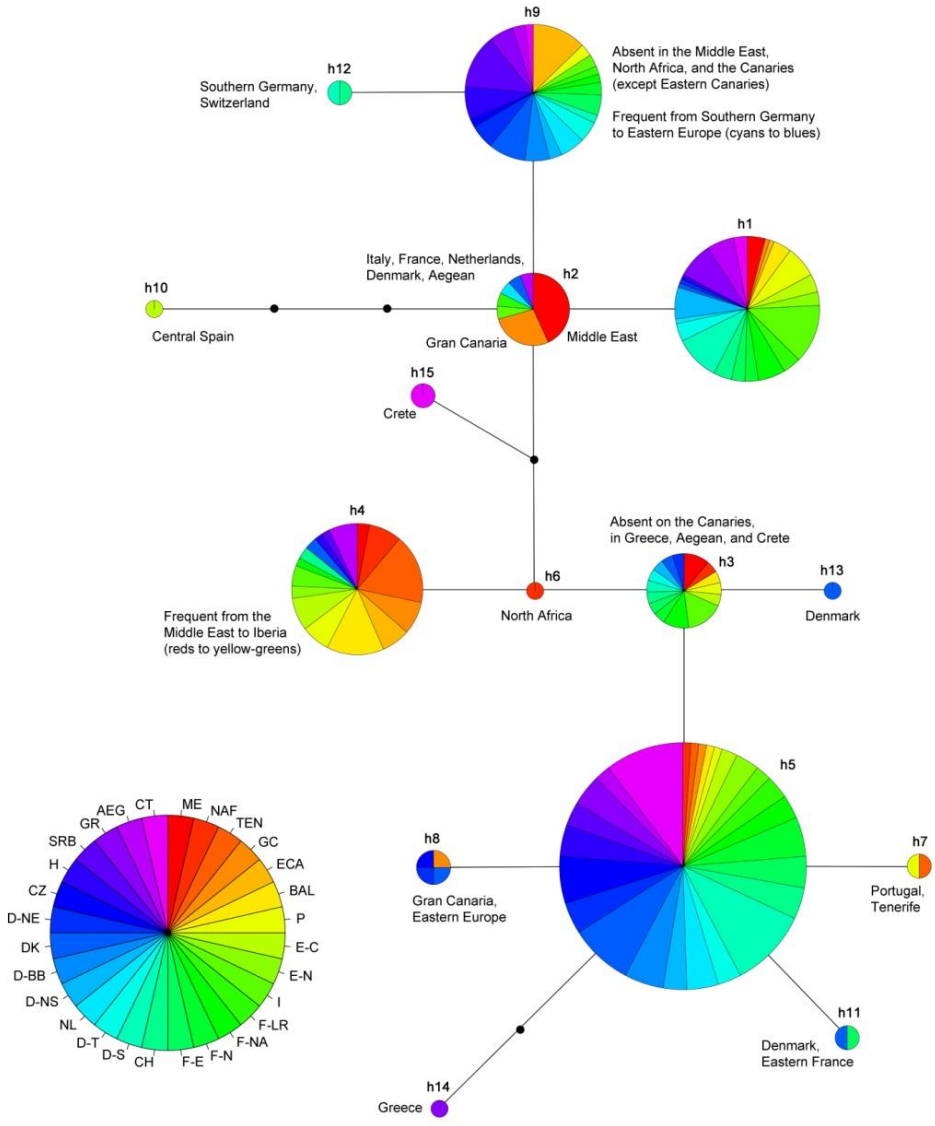

Figure S8. Mitochondrial ND6 haplotype network in the barn owl. The surface of the pies is proportional to haplotype frequencies. Black dots represent unobserved haplotypes.

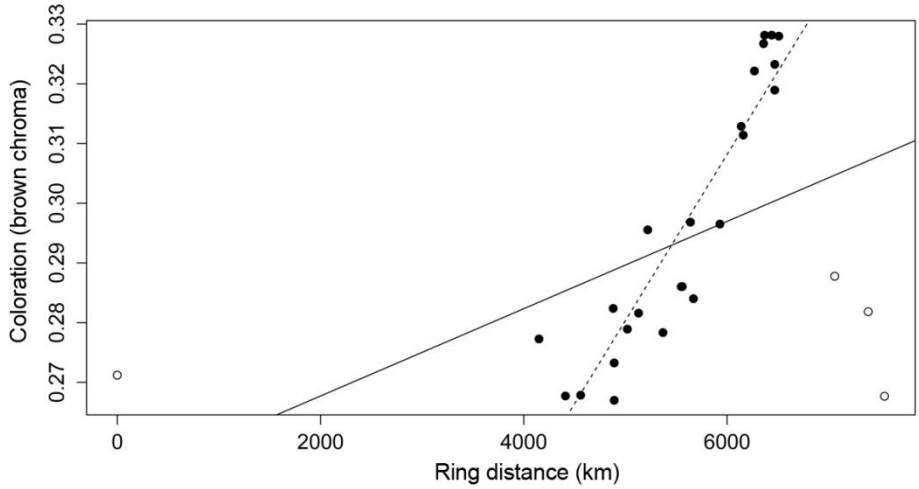

Figure S9. Spatial distribution of populations' mean coloration along the circum-mediterranean ring in the barn owl. Ring distance is the population's distance from the Middle East. Solid line: Regression across all populations (R^2=0.22, t=2.69, p=0.012). Dashed line: Regression with populations from the Middle East, Greece, Aegean, and Crete (open circles) excluded (R^2=0.85, t=11.23, $p<10^{-9}$).

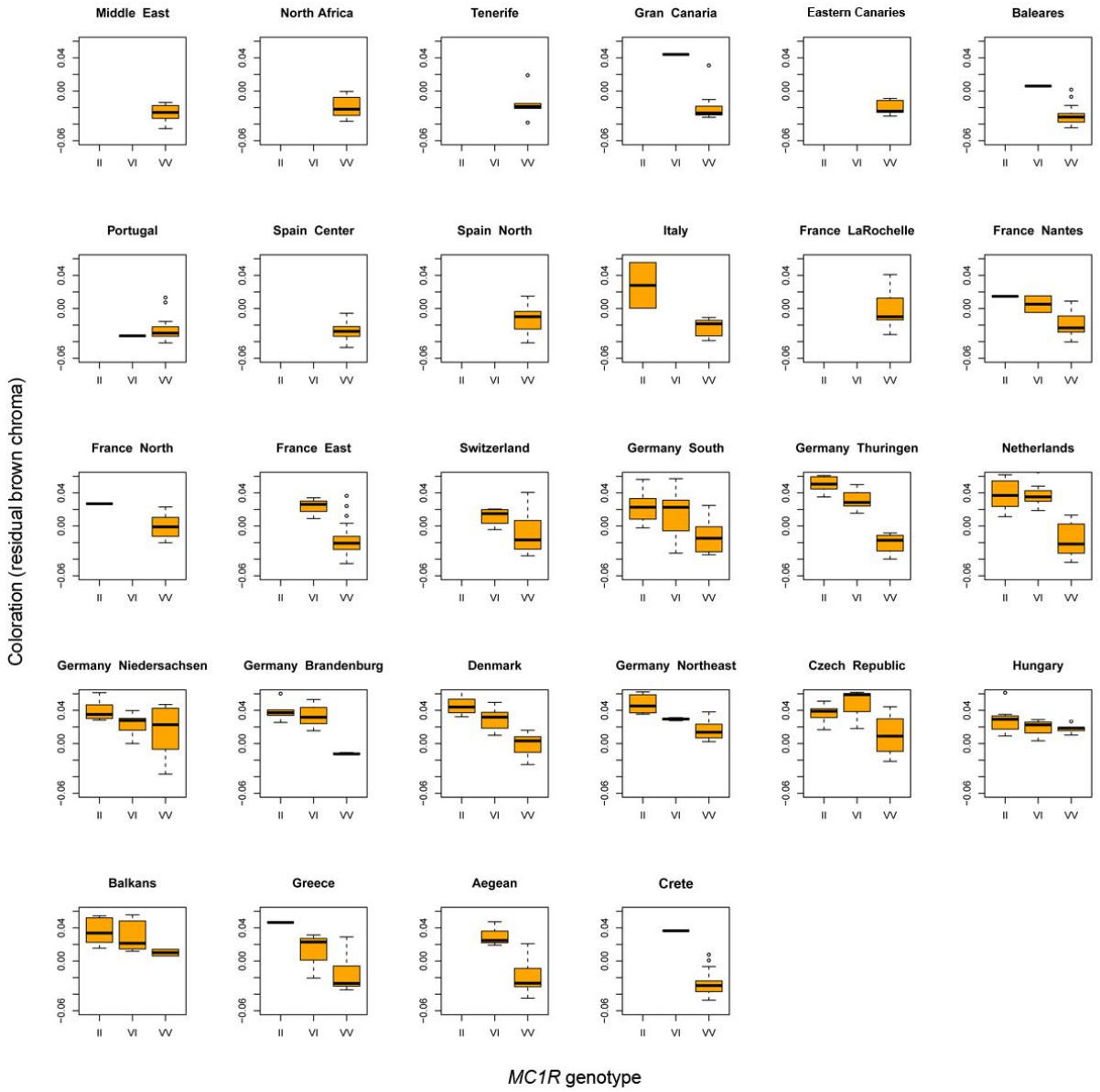

Figure S10. Population-wise relationships of coloration with MC1R genotype in the barn owl. Coloration is shown as residual brown chroma after correcting for differences between sexes.

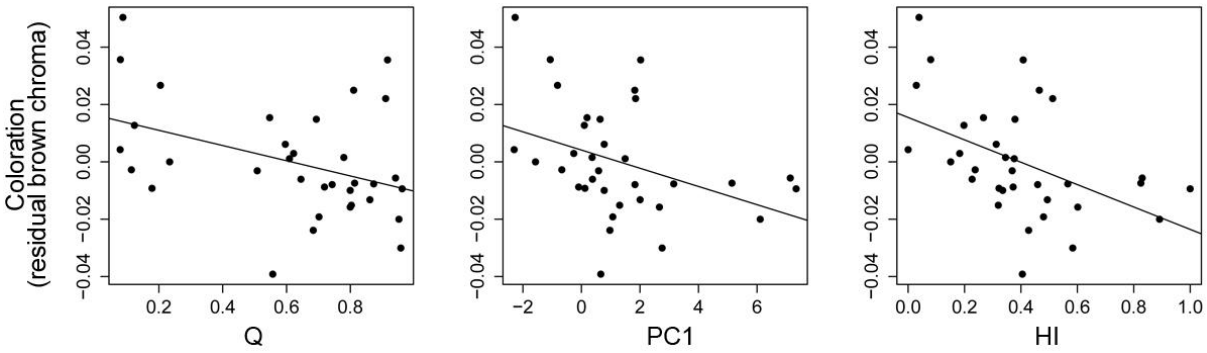

Figure S11. Correlations of coloration with genetic ancestry in the secondary contact zone (Greece and Aegean). Coloration is given as residual brown chroma after correcting for sex differences and MC1R genotype. Genetic ancestry was measured by admixture proportions (Q), the first axis of an individual-based PCA (PC1), and with the hybrid index (HI).

Table S1. Population sample sizes, sample sizes for genetic markers and color phenotypes, and ring distance from the Middle East.

Population	Abbrev.	Msat	ND6	MC1R	Color	Ring distance (km)
Middle East	ME	32	15	32	15	0
North Africa	NAF	19	10	12	18	4150
Tenerife	TEN	26	14	20	10	5130
Gran Canaria	GC	16	15	16	11	5020
Eastern Canaries	ECA	17	14	16	7	4890
Baleares	BAL	29	14	28	29	4890
Portugal	P	30	15	26	30	4410
Spain Center	E-C	20	15	20	20	4560
Spain North	E-N	11	11	11	11	4880
Italy	I	25	22	22	14	5550
France LaRochelle	F-LR	13	12	13	13	5220
France Nantes	F-NA	28	15	28	28	5370
France North	F-N	15	15	15	15	5640
France East	F-E	28	15	28	28	5670
Switzerland	CH	27	16	27	27	5560
Germany South	D-S	37	30	37	37	5930
Germany Thuringen	D-T	19	14	19	19	6140
Netherlands	NL	30	15	30	30	6160
Germany Niedersachsen	D-NS	30	15	30	30	6270
Germany Brandenburg	D-BB	27	15	26	27	6360
Denmark	DK	37	30	37	37	6440
Germany Northeast	D-NE	21	15	21	21	6510
Czech Republic	CZ	20	14	20	20	6370
Hungary	H	32	15	32	16	6470
Balkans	SRB	28	15	18	21	6470
Greece	GR	24	19	17	21	7060
Aegean	AEG	22	16	19	20	7390
Crete	CT	61	25	51	51	7550
Total		*724*	*456*	*671*	*626*	

Table S2. Microsatellite multiplex sets.

Multiplex	Locus	Dye	Final Conc. [µM]	Reference
Multiplex 1	Ta-206	FAM	0.450	Burri *et al.* (2008)
	Ta-207	NED	0.052	
	Ta-210	HEX	0.105	
	Ta-216	FAM	0.135	
	Ta-306	NED	0.165	
	Ta-308	HEX	0.075	
Multiplex 2	Ta-218	HEX	0.178	Burri *et al.* (2008)
	Ta-219	NED	0.110	
	Ta-220	FAM	0.110	
	Ta-304	HEX	0.041	
	Ta-414	HEX	0.275	
Multiplex 3	Ta-204	HEX	0.250	Burri *et al.* (2008)
	Ta-214	FAM	0.500	
	Ta-305	FAM	0.500	
	Ta-310	NED	0.25	
	Ta-413	NED	0.25	
Multiplex 4	Ta-202	FAM	0.250	Burri *et al.* (2008)
	Ta-212	DYO630	1.000	
	Ta-215	FAM	1.000	
	Ta-402	NED	0.250	
	Ta-408	HEX	0.50	
Multiplex 5	FEPO42	FAM	0.250	Klein *et al.* (2009)
	54f2	NED	0.250	
	Tgu06	HEX	0.500	
	Calex-05	DYO630	0.500	
	RBG18	FAM	0.750	
	Oe053	HEX	1.00	

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General discussion

In this thesis, I studied the evolution and maintenance of the genetically based colour cline observed in the European barn owl (*Tyto alba*) in unprecedented details. I was able to show that the post-glacial history assumed for European barn owls until the beginning of my thesis was wrong and that the colour cline did not evolve by secondary contact after the last ice age as suggested by Voous (1950) and retaken by all authors after him (eg. Matics *et al.* 2005; Roulin 2003).

With the first populations' genetics dataset covering more than a few populations for this model species, we were able to redefine barn owl post-glacial population history for the Western Palearctic. During the ice age, European barn owls were confined in the Mediterranean basin and maybe even only in its eastern part. As all barn owl of the Mediterranean basin present very few variation today, the colour variation was probably also minor at that time. After the ice age most of Europe was recolonized by the species from the Iberian Peninsula. During or just after this colonisation, the colour polymorphism that we observe today, evolved mainly by the mutation of a single amino acid of one of the "light" haplotype of the *MC1R* gene into a "dark" haplotype. We were also able to show that the differences in colour and on the frequency of the *MC1R* allele are maintained despite high gene flow by selection for local adaptation. Probably at the same time, a second minor colonisation occurred from the eastern Mediterranean basin toward Greece by light barn owls. Those two colonisation routes meet back in Greece (and maybe more extensively toward the black sea) in a secondary contact zone where we observed dark and light barn owls and where we have some evidence of limited introgression between these two colour morphs. If the reproductive isolation between dark and light barn owls is complete in

Greece, the European barn owl will constitute a perfect example of a ring species (Irwin 2009; Irwin *et al.* 2001b; Martens & Packert 2007).

In the next sections of this discussion, I will first present and discuss the main missing pieces of the post-glacial history of European barn owl and the evolution of the colour cline. I will then present the major perspectives that the western Palearctic barn owls offer for our understanding of the interaction between range expansion, local adaptation and ultimately speciation.

The selective agent behind European barn owl colour variation: the missing piece.

The main missing piece for the understanding of the European barn owl colour variation is to identify the selective agent that drives either the colour variation itself or part of its underlying genetics.

Our European dataset is however unfortunately probably not the best way to approach the question. Many environmental variables show a south-west/north-east gradient across Europe and it would be difficult to get a definitive answer of which one might be the selective agent for the colour variation (see discussion in chapter 1). Furthermore, the number of potential selective forces acting on colouration is enormous and their comprehensive review is far beyond the scope of this discussion. Broad categories include sexual selection, natural selection on optical properties (for example camouflage) or non-optical properties (for example the roles of melanins as mechanical strengthening) (reviews include Andersson 1994; Bortolotti 2006; Hill 2006; McGraw 2006; Senar 2006). On the top of direct selection on colour itself, it must not be overlooked that the colour

variation might arise due to its correlation with other traits or for pleiotropic effects of the gene determining colour (Ducrest *et al.* 2008).

I will first review what is known about pheomelanic colour variation in the barn owl and hypothesise the selective agent behind European colour variation. And second highlight a research agenda that might be most rewarding regarding this question.

Many ecological characteristics of barn owls have been shown to covary with its pheomelanic colour variation (the variation from white to rufous-brown of its underparts): in Switzerland the diet varies among the different colour phenotypes (Roulin 2004) and this relationship has also been observed in Israeli barn owls even if the colour variation is far less pronounced (Charter *et al.* 2012). In the long term population study in Switzerland, a link between colour and breeding rate has been found as well as with the growth rate of the owlet (Roulin & Altwegg 2007; Roulin *et al.* 2008). A link between colour variation and dispersal propensity has also been described (van den Brink *et al.* 2012). Even if the synthesis of all those scattered observations is still lacking, this shows that small ecological differences exists among barn owl colour variants. This is consistent with the observation of some evidence for local adaptation, even in Switzerland at a small spatial scale (Dreiss *et al.* 2012). The claim by Galván and Alonso-Alvarez (2011) that natural radioactivity might be the selective agent behind colour variation is interesting but deserves a proper analysis including more than a single environmental variable.

One level of complexity might be added by the correlation among colour traits and by the putative pleiotropic effects of the genes underlying the colour variation. Those two possibilities might be partially met in the barn owl. Roulin have shown that pheomelanic colouration in the barn owl is correlated with eumelanic variation (Roulin 2003) and the eumelanic variation is under directional selection in female barn owl (Roulin *et al.* 2010). The

second possibility of having the genes underlying the phenotypic variation showing pleiotropic effects for other functions could also bet met in the barn owl. The *MC1R*, which seems to be at least one of the major driver of the colour variation has recently be shown to be involved in many other functions (Ducrest *et al.* 2008).

Our understanding of the European barn owl colour cline will be a lot more complete with the identification of the selective agents behind the colour variation or some of its underlying genes (*MC1R* and maybe some other genes still to be identified). My guess regarding this question is that the European colour cline is driven by metabolic effects linked to colour variation and to winter survival. Several lines of evidences may indicate this direction: the south-west/north-east colour cline corresponds to a marked cline in continentality which has a direct link to winter harshness; one of the major driver of barn owl population dynamics is the sensibility of the species to winter harshness (Altwegg *et al.* 2006); a link between pheomelanic colouration and winter survival has been shown for several species including three owl species (Galeotti *et al.* 2009; Karell *et al.* 2011; Mosher & Henny 1976; Sirkia *et al.* 2010). As mentioned earlier, the European dataset suffer the problem of having many environmental variables correlated on the south-west/north-east axis, I thus think that using long-term population studies dataset is far more promising to identify the selective agent behind the colour variation. The first two tests that I would propose is (1) to look at the survival as a function of colour and winter harshness as in Karell (2011) and (2) to look as the frequency of the *MC1R* alleles as a function of winter harshness. Preliminary data from our lab regarding the second hypothesis seems to show a link between the temperature in March and the frequency of the "dark" *MC1R* allele the following spring (Alexandre Roulin, pers. comm.). One potential confounding factor with this

approach is that the Swiss population is far from being isolated and that it might be difficult or impossible to disentangle effect from natural selection and effect from immigration.

The identification of the selective agent behind European colour variation remains the big question for understanding the evolution of the colour cline. Time has probably come to synthetize all data and evidence that have been collected in the last decades and try to decipher or at least identify hypotheses to test regarding this question.

The European barn owl model system: perspectives

The genetic determinism of the colour variation

The link between phenotypic variation, its underlying genetics and fitness differences in natural populations has been a big challenge for a long time (Ellegren & Sheldon 2008; Stapley *et al.* 2010). Thanks to technological advancements, more and more example where those links start to be well understood are accumulating (eg. Barrett & Hoekstra 2011; Ellegren & Sheldon 2008; McNiven *et al.* 2011; Savolainen *et al.* 2013; Stapley *et al.* 2010). Colour variation is no exception to this trend (eg. Hoekstra 2006; Hofreiter & Schoeneberg 2010; Hubbard *et al.* 2010; Kronforst *et al.* 2012; Mundy 2006). Variation in melanic colouration are ubiquitous in nature and, when known, the genetic architecture behind those colour variation presents a tremendous lability (McGraw 2006).

However, the *MC1R* gene has been found to be involved over and over again in colour variation of birds, but also other vertebrates' taxa. It was thus not very surprising that at least part of the colour variation in Barn owl was also determined by this gene (see chapter 3).

However, the link between *MC1R* and colour in Europe seems far to be one to one. On average, the *MC1R* gene explains along with the sex and genetic structure, more than

60% of the colour variation in the Western Palearctic (chapter 3), but some populations do not conform to this rule: in Hungary for example the link between *MC1R* genotype and colour is very restricted (Chapter 3, figure S8). Another interesting observation is that there is a link between neutral genetic ancestry and colour in Israel despite no variation on the *MC1R* gene, which show that some other loci that have introgressed from north-eastern Europe must be involved in the colour variation in Israel (Sylvain Antoniazza, pers. obs.). These observations show that the genetic architecture of the colour variation in Europe is far more complex than just the existence of two different alleles of the *MC1R* gene, even if this gene already permits to explain a significant part of the colour variation in Europe.

Another interesting aspect offered by barn owls is that colour clines are replicated independently on several continents and in several taxa (Roulin *et al.* 2009). This opens the possibility to test whether the genetic determinism of the colour variation evolved differently or in the same way in the different colour clines.

How strong is selection on colour and on the *MC1R* gene?

The big differences between differentiation on the colour itself and on the *MC1R* gene and neutral genetic markers show that selection is involved in the maintenance of the colour cline and on the maintenance of allele frequencies differences of the *MC1R* gene. This suggests that selection on colour and on its underlying genes might be strong (chapter 3, fig. 4). Estimating the strength of the selection on colour and on the *MC1R* gene is one of the question that must be tackled in a near future to better understand the model system. A few attempts have been made but answering the question in a satisfactory way seems challenging.

One attempt has been made following Mullen and Hoekstra (2008). These authors have used classical cline theory to estimate the selection acting on a colour cline that did not result from secondary contact (on the contrary of most application of "cline theory"). In this case, the application seemed successful and to make sense. However, the application in the very similar barn owl case seems less promising. With the R package HZAR (Derryberry *et al.* 2013), it was only possible to fit a cline between south-west Europe and north-east Europe for the *MC1R* gene allele frequencies (and not for the colour phenotypic variation). Using the cline equation for the gradient model, the selection coefficient found for the *MC1R* gene is of $8.51*10^{-5}$. This value seems very low and I have the feeling that this is not very close to the actual strength of selection. Based on the project of Ricardo Kanitz to estimate selection on a trait subject to local adaptation in a colonisation context (Kanitz et al., in prep.), the estimated selection coefficient might be close to 0.3. These two values sets to extrems limits to the most likely value of the selective coefficient. One reason for the cline model to perform poorly might be that those models have been designed for equilibrium cases where the action of migration, drift and selection is at equilibrium. This is probably why cline theory performs very well in hybrid zones where those equilibrium conditions are generally met. The barn owl case seems to be far from this ideal equilibrium conditions. The historical colonisation from the Iberian Peninsula has left strong traces in the genetics of the European barn owls for neutral genes and potentially for gene under selection too. On top of this historical colonisation, the dynamics of extinction/recolonisation due to cold winter might also play an important role and move the model system away from equilibrium conditions (see below part on recurrent adaptation).

I think that the most promising approach to estimate the strength of selection on the *MC1R* gene and on colour itself is to use a simulation approach. The simulation basis that

has been set up for the neutral ABC study (see chapter 2) seems to make a perfect basis for such a study. One potential limitation might be computer speed. The model that we used with coalescence takes several hours to run forward in time with genes under selection. Some shortcuts must be found to be able to perform a true ABC approach to estimate selection in this system. One possibility might be to first study selection only on the two *MC1R* alleles. For such a simple genetic architecture, coalescent approach have already been proposed (Ewing & Hermisson 2010). It might even also well be that forward in time simulation are short enough to be considered and Quantinemo directly use to tackle the question (Neuenschwander *et al.* 2008). This is not a very short and simple project, but I am sure that this approach will be rewarding, both to understand the barn owl model system and in general.

There is still a bit of work to be able to find a good model to estimate how strong is the selection on the color of the European barn owl and on the *MC1R* gene, but many first steps have already been made and I am convinced that this could be a rewarding way to go.

Evolution during colonisation

Despite the fact that many (if not all) species regularly experience range expansion and contraction as well as range shift mostly as a result of past and current climatic changes, the genetic consequences of these demographic processes have not been investigated in detail (Excoffier *et al.* 2009). One reason might also be that range expansion, contraction and shift are complex processes and that they could essentially only be studied by simulation approaches (but see Slatkin & Excoffier 2012 for a first analytical derivation).

The effect of range expansion on the fate of neutral genetic variation has received most of the attention and several recently described phenomena, like gene surfing, start to be well known and understood (Edmonds *et al.* 2004; Klopfstein *et al.* 2006). However, range expansions may have many other consequences like asymmetrical gene flow (Petit & Excoffier 2009), or spatial sectorization of the genetic variation (Excoffier & Ray 2008). These results indicate that many population genetic patterns that are generally attributed to selective processes should be interpreted with caution and if possible the main neutral demographic hypothesis should be ruled out by simulation. This is what we did in the second chapter of this thesis, where we show that the probability that the barn owl colour cline evolved by purely neutral surfing is extremely low. There is probably many examples in the literature of study where an alternative neutral demographic explanation has not been rigorously tested; for the most obvious, critical answer have been made (eg. Currat & Excoffier 2011; Vasemägi 2006). Even today many studies do not evaluate this issue. But there is also good example where this issue has been addressed carefully: White et al. (2013) have used three independent colonisation fronts to investigate patterns of selection in the bank vole, Kujala and Savolainen developed a basic (non-spatial) demographic model in their study of a clinal variation in scots pine (Kujala & Savolainen 2012).

Following the discovery that range expansion might have effects on neutral genetic variation that where not foreseen before their careful investigations, generally by simulations, some other effects of range modification have been studied. The most straightforward is the effect of range contraction and range shift that have been addressed by Arenas (2012). Beside effects on neutral genetic variation, these demographic effects have for sure also an effect on loci that are under selection. Given that many of the range modification occur by definition along major ecological axes, the interaction between those

demographic effect and selection, for example for local adaptation, are of key importance. These topic only started to be addressed (Excoffier *et al.* 2009; Lehe *et al.* 2012; Peischl *et al.* 2013; Travis *et al.* 2007).

The European Barn owl provides a perfect model system to investigate these questions. We show in the second chapter how this species colonised western Europe from the Iberian Peninsula by a major range expansion. We also showed in the first and third chapters, how a colour cline evolved during or after this colonisation. All these informations and the neutral demographic models developed in the second chapter permit to investigate in many details the interaction between the colonisation of western Europe and the establishment of the colour cline, as well as its genetic determinants like the *MC1R* gene.

One last interesting feature that can be studied in the Barn owl is the possibility to discover recurrent adaptation in this species. The fact that the barn owl experiences strong population fluctuation because of its sensitivity to winter harshness could drive such a pattern (Altwegg *et al.* 2006; Marti & Wagner 1985; Massemin & Handrich 1997). The ornithological literature stipulates that these population crashes are compensated by emigration from southern population. This opens the possibility that south-western barn owl regularly colonise north-eastern Europe. This would imply recurrent selection on these emigrants. The system would have to be studied in far more detail to carefully evaluate this possibility, but Barn owl would at least offer the perfect conditions do have such recurrent adaptation (strong dispersal abilities, strong selection for local adaptation and regular local population crashes).

Local adaptation in an homogenous background

Examples from natural population that shows that some local adaptation exists between populations of many species are accumulating at a quick rate (Hereford 2009). Our view of local adaptation shifted from something believed to be rare to an ubiquitous process (Hereford 2009). It was also realized that even if a trade-off between local adaptation and gene flow exists (Räsänen & Hendry 2008), local adaptation might develop even in the presence of gene flow. The study of this process – differentiation in the presence of gene flow – is also of key importance for the study of the speciation process. The literature on ecological speciation, where speciation results from adaptation to various ecological niches within a single species, has grown enormously in the last two decades (Nosil 2012) and example of speciation gene flow might not be as rare as previously thought (Feder *et al.* 2012; Nosil 2008).

The evolution of the European barn owl cline is also very interesting in this context. In the classical view of its evolution, Voous (1950) postulated that the two colour morphs evolved in allopatry, which means in the absence of gene flow. In our new scenario, the local adaptation of barn owl colour evolved during or after the colonisation of the whole European continent by Barn owl coming from the Iberian Peninsula. This scenario suggests that the local adaption on colour arise with probably high level of gene flow in a very homogenous genetic background. For example, this can be seen in the supplementary figure S2 of the third chapter of this thesis. The patterns that can be seen in the *MC1R* tree are opposite to the pattern seen in the neutral genetic tree, in other words all the differentiation on the *MC1R* occur among barn owl that are very similar in their neutral genetic background.

The European barn owl thus represents one more example of the high level of differentiation that can be attained despite high levels of gene flow. The barn owl system is

also very interesting in the sense that the white to rufous-brown colour cline from Iberia to north-eastern-Europe occurs among very similar Barn owls but that the rufous-brown to white colour cline that occur from north-eastern-Europe to the middle-east occur among the most different barn owls (on a neutral genetic point of view). The European barn owl model thus provides some more opportunities to study the local adaptation process in more details.

Studying speciation in space and time

For several decades, evolutionary biologists have considered that speciation could not occur without some form of geographical isolation between the incipient species (Bolnick & Fitzpatrick 2007; Mayr 1963). This view has drastically changed in the last decade, thanks to both empirical examples (Barluenga *et al.* 2006; Savolainen *et al.* 2006; Schliewen *et al.* 1994; Sorenson *et al.* 2003) and theoretical models (see Bolnick & Fitzpatrick 2007 for a review), showing that sympatric speciation can and does occur. One key mechanism involved is divergent selection towards local optima (Coyne & Orr 2004; Schemske 2010). This mode of selection can be so pronounced that it might cause speciation despite high levels of gene flow (Barton 2010; Nosil 2008). In reality, sympatric and allopatric speciation represent the two ends of a continuum from speciation with maximal gene flow (panmixia) to speciation with zero gene flow. Most natural examples will fall somewhere between these two extremes, thus a more integrated view including measures of dispersal, gene flow and selection as well as accounting for the spatial and historical contexts is needed to study the speciation process (Feder *et al.* 2013; Fitzpatrick *et al.* 2009; Mallet *et al.* 2009). The historical context is a key element: when a group of individuals at the same geographical

location show signs of incompatibility, it might be because they have diverged on site, or because they came into contact after being separated for some times. Understanding the speciation process therefore requires a deep understanding of the biogeographical origin of a group of sister species to apprehend the relative role of allopatric and sympatric evolution in speciation. Ring species in general and the Barn owl model system that I presented in this thesis provide ideal model systems to study these questions.

In the European barn owl model system, one important remaining question is the exact taxonomic status of the two colour morphs coming into secondary contact in the Balkans. Are those two taxa showing only slight differentiation for colour-phenotypes and a handful of genetic markers as in willow warbler (Bensch *et al.* 2009) or do they present more restricted gene flow and merit a species status as in for example greenish warblers (Irwin *et al.* 2001a). In continental Europe, two subspecies of barn owl where generally defined based on colour variation, the light breasted *Tyto alba alba* subspecies and the dark breasted *Tyto alba guttata* subspecies. Following Voous (1950), they were always considered to have diverged in allopatry during the last ice age, but we have seen that this scenario has to be completely revised according to the results presented in this thesis.

In the western part of the distribution (one study in Switzerland, one in eastern France and one in Hungary), the colour morphs show no sign of assortative mating (Baudvin 1986; Matics *et al.* 2002; Roulin 1999). However, if the two colour morphs that come into secondary contact in the Balkan Peninsula merit a species status, they might present some form of pre-mating isolation and may show assortative mating. Unfortunately, only sparse data are available for this area. My research agenda regarding this question would be in two steps (1) I would try to better define the structure of the secondary contact zone between dark barn owls from the north and light barn owl from the south. Our data show that both

light and dark barn owl occur in Greece but we do not have enough data to understand where light barn owl does not occur anymore when moving north, except that we only found dark barn owl in north of the Balkans. We also have cues that both light and dark barn owls occur, for example, in Romania (Sylvain Antoniazza, pers. obs.). I would thus predict a secondary contact zone running from the Adriatic Sea towards the black sea. It is even possible that a secondary contact zone also exist in Italy (Barn owls are known to be light in southern Italy, Cramp 1985). I would thus recommend setting up a sampling scheme spanning at least all the Balkans. This should permit to decipher the structure of the secondary contact zone between dark and light barn owls and set basis for a deeper understanding of the level of gene flow that occur between the two colour morphs in this region. This sampling would also permit to take advantage of recent technical progress in high-throughput sequencing and would give the perfect settings for the search of incompatibility gene involved in post-mating isolation (Feder *et al.* 2013; Gompert & Buerkle 2011) (2) After having identified the zones where both light and dark barn owl occur, I would set up one or several populations monitoring, to study for example the mating behaviour. It would also permit to see if barn owls present ecological differences related to colour differences when they occur in sympatry. Such pattern was found in Switzerland, but the differences where subtle (Dreiss *et al.* 2012). We can imagine that the differences might be more pronounce between barn owls that are more divergent.

We have seen in this thesis that the barn owl model system provides wonderful settings to study the interaction between historical factors (e.g. range expansion, secondary contact), ecological characteristics (e.g. dispersal, local adaptation) and the process of divergence and ultimately speciation. If we were able to obtain a clear view of many aspects of the evolution and maintenance of the European barn owl colour cline, many others have

still to be deciphered. The main questions that should be tackled in a near future include: what is the selective agent that drives the colour differences in the barn owl colour cline? How strong selection should be to maintain dramatic differences in the frequency of the alleles of the *MC1R* gene despite no neutral genetic differences? How reproductively isolated are light and dark barn owl in Greece?

Beside those question that could maybe be answered quite soon, a deeper understanding of the whole model system from metabolic differences between the colour variants toward the speciation process at the secondary contact constitute an ambitious but probably fruitful research agenda.

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