Evolutionary Insights Into Felidae Iris Color Through Ancestral State Reconstruction

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Abstract

There have been almost no studies with an evolutionary perspective on eye (iris) color, outside of humans and domesticated animals. Extant members of the family Felidae have a great interspecific and intraspecific diversity of eye colors, in stark contrast to their closest relatives, all of which have only brown eyes. This makes the felids a great model to investigate the evolution of eye color in natural populations. Through machine learning cluster image analysis of publicly available photographs of all felid species, as well as a number of subspecies, five felid eye colors were identified: brown, hazel/green, yellow/beige, gray, and blue. Using phylogenetic comparative methods, the presence or absence of these colors was reconstructed on a phylogeny. Additionally, through a new color analysis method, the specific shades of the ancestors' eyes were reconstructed to a high degree of precision. The ancestral felid population was found to have brown eyed individuals, as well as a novel evolution of gray eyed individuals, the latter being a key innovation that allowed the rapid diversification of eye color seen in modern felids, including numerous gains and losses of different eye colors. It was also found that the loss of brown eyes and the gain of yellow/beige eyes is associated with an increase in the likelihood of evolving round pupils, which in turn influence the shades present in the eyes. Along with these important insights, the unique methods presented in this work are widely applicable and will facilitate future research into phylogenetic reconstruction of color beyond irises.

Introduction

Eye (iris) color is one of the most conspicuous and varied traits between animals with irises. To date, much of the work investigating eye colors has focused on humans. This is not surprising, given how stark differences in human eye color can be, even between close relatives. It is known that human eye colors differ due to a relatively small number of genes that act on the amount and quality of melanin in the eye (White and Rabago-Smith 2011). In addition, the diversity of human eye colors, ranging from brown to green to blue, has been attributed to sexual selection (Frost 2006). Such intraspecific eye color variation has been described as rare amongst animals, apart from artificially selected domesticated animals (Negro *et al.* 2017). However, even when just regarding eye color differences between species, few hypotheses have been tested and very little is known about the adaptive benefits or evolutionary history of eye color, particularly in a natural context. A major reason for this lack of study is laid out in Negro *et al.* (2017): the belief to this point has been that "in those species for which the ancestor is now extinct . . . we can chart the diversification of eye color over time only by retrieving ancient DNA from remains".

As with most groups, little work has been done understanding the eye colors of members of the family Felidae. Behavioral traits have been correlated to the eye colors of domesticated cats (*Felis catus*), but the wild felids have been largely left unstudied in this regard (Wilhelmy *et al.* 2016). This is surprising, given that the closest relatives to the felids, such as linsangs, hyenas, and mongooses, all have brown eyes, with little inter- or intraspecific variation between them (Johnson *et al.* 2006). In contrast, the felids have a wide diversity of eye colors within and between them, even without counting *F. catus*. Although this trait is apparent by simply looking at members of each species, it has never been studied in an evolutionary context. It is of particular interest to

reconstruct the ancestral state of eye color in the felids, because such reconstructions can shed light on the history of the trait, interrogating how and why its current forms exist. Such analyses are vital for broadly increasing knowledge of evolution in a natural context, particularly since eye color is not retained in fossils, nor in most preserved specimens. This has been done to great effect in owls; however, only "light" and "dark" eyes were considered in the analysis, not specific eye colors (Passarotto *et al.* 2018).

Here, we present the first phylogenetic comparative analysis of eye color. We examine representatives from every extant felid species, as well as a number of subspecies, using a new quantitative color analysis method, to try and solidify a categorization of eye color for these groups. Using this data, we have reconstructed the eye colors of the ancestors of the felids at all phylogenetic nodes, as well as tested their correlations with environmental and morphological data, to better understand the diversification of felid eye colors and demonstrate that phylogenetic investigations into eye color are not only possible, but fruitful.

Materials and Methods

Data set: In order to sample all felid species, we took advantage of public databases. Images of individuals from 40 extant felid species (all but *Felis catus*, excluded due to the artificial selection on eye color in domesticated cats by humans), as well as 13 identifiable subspecies and the banded linsang as an outgroup (*Prionodon linsang*), were found using Google Images and iNaturalist using both the scientific name and the common name for each species as search terms. This approach, taking advantage of the enormous resource of publicly available images, allows access to a much larger data set than in the published scientific literature or than would be possible to obtain *de novo* for this study. Public image-based methods for character state classification have

been used previously, such as in a phylogenetic analysis of felid coat patterns (Werdelin and Olsson 1997). However, this approach does require implementing strong criteria for selecting images.

Criteria used to choose images included selecting images where the animal was facing towards the camera, at least one eye was unobstructed, the animal was a non-senescent adult, and the eye was not in direct light, causing glare, or completely in shadow, causing unwanted darkening. The taxonomic identity of the animal in each selected image was verified through images present in the literature, as well as the "research grade" section of iNaturalist. When possible, we collected five images per taxon, although some rarer taxa had fewer than five acceptable images available. In addition, some species with a large number of eye colors needed more than five images to capture their variation, determined by quantitative methods discussed below. Each of the 54 taxa and the number of images used are given in Supplementary Table 1.

Once the images were selected, they were manually edited using MacOS Preview. This editing process involved choosing the "better" of the two eyes for each felid image (i.e. the one that is most visible and with the least glare and shadow). Then, the section of the iris for that eye without obstruction, such as glare, shadow, or fur, was cropped out. An example of this is given in Figure S1. This process resulted in a data set of 269 cropped, standardized, felid irises. These images, along with the original photos, can be found in the Supplementary Material.

Eye color identification: To impartially identify the eye color(s) present in each felid population, the data set images were loaded by species into Python (version 3.8.8) using the Python Imaging Library (PIL) (Van Rossum and Drake 2009; Clark 2015). For each image, the red, green, and blue (RGB) values for each of its pixels were extracted. Then, they were averaged and the associated hex color code for the average R, G, and B values was printed. The color associated

with this code was identified using curated and open source color identification programs (Aerne 2022; Cooper 2022). This data allowed the color of each eye in the data set to be correctly identified, removing a great deal of the bias inherent in a researcher subjectively deciding the color of each iris.

Eye colors were assigned on this basis to one of five fundamental color groups: brown, hazel/green, yellow/beige, gray, and blue. To ensure no data was missed due to low sample size, the first 500 Google Images, as well as all the "research grade" images on iNaturalist, were viewed for each species. Any missed colors were added to the data set. This method nonetheless has a small, but non-zero, chance to miss rare eye colors that are present in species. However, overall, it provides a robust and repeatable way to identify the general iris colors present in animals.

In addition, if, for a given species, one or two eye colors were greatly predominant in the available data online (>80% for one or ~40% for both, respectively), they were defined as being the most common eye color(s). With this assessment, the phylogenetic analysis below could be carried out both with all recorded eye colors and using only the most common eye colors, thereby assuring that rare eye colors did not skew the results. All the eye colors present for each species are displayed in Figure 1.

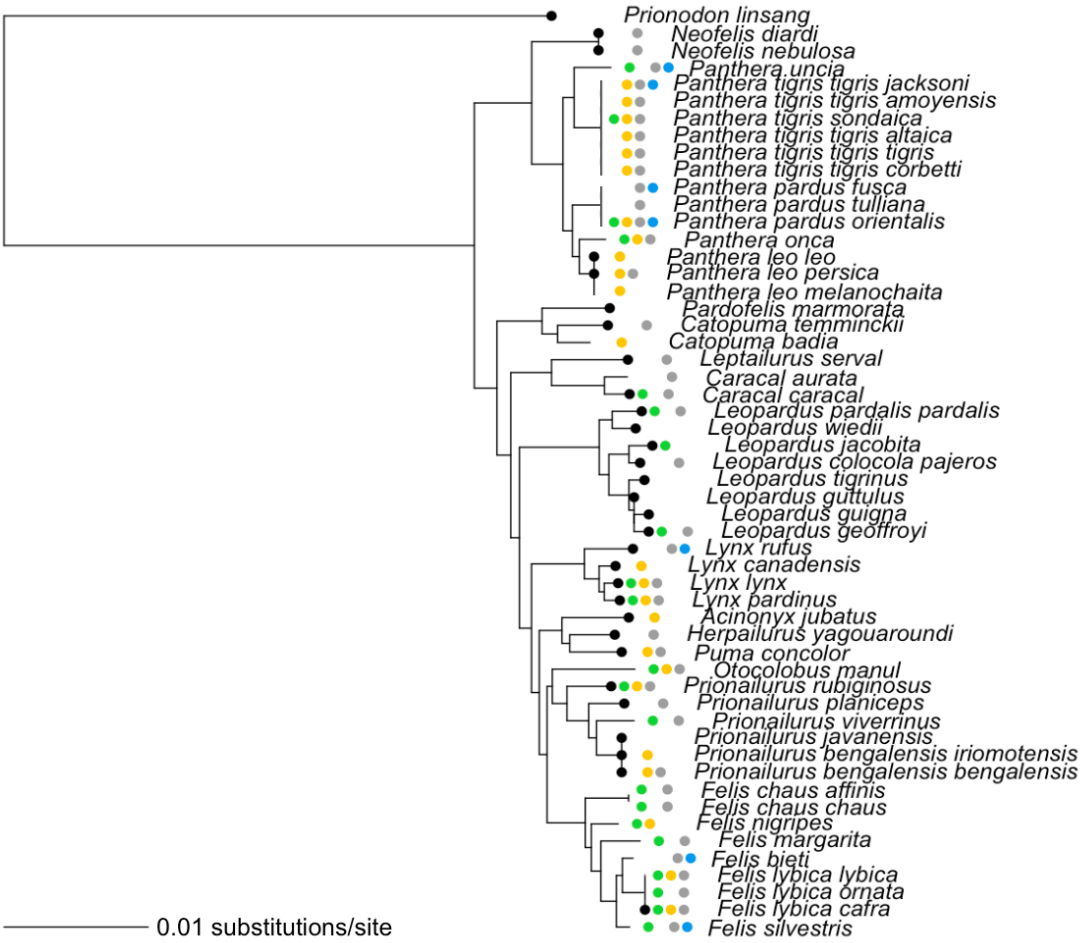

Figure 1: The "short" phylogenetic tree, modified from Johnson et al. (2006), with iris color data. Dots next to tips represent the presence of each eye color (black dot: brown eyes, green dot: green/hazel eyes, yellow dot: yellow/beige eyes, gray dot: gray eyes, blue dot: blue eyes). No dot for a given color represents an absence of that color from the respective taxa. Graph created using plot.phylo() in ape.

Shade measurements within each color group: For each species, the images were sorted into their groups by assigned color. For each group, RGB values for each pixel in each image were again extracted, resulting in a three dimensional data set. This was reduced to two dimensions using Uniform Manifold Approximation and Projection (UMAP) (McInnes *et al.* 2018). The graph for each image was then analyzed using k-means clustering through the package scikit-learn (version 1.2.0) (Pedregosa *et al.* 2011). The number of clusters (k), indicating the number of distinct shades of color in the iris of each animal, was determined using elbow plots.

After this was done for all images in the group, the k values were averaged and each image was clustered using the average k value, rounded to the nearest integer. This was done to standardize within groups, avoid confounders based on lower quality images, and allow for comparative analysis. After this, the average RGB values for each cluster for each image were calculated. Then, the clusters were matched up based on similarity. To do this, one image from the group had its clusters labeled in order (if there were three clusters, they would be 0, 1 and 2). Then, another image from the group would have the distances in 3D space between each of its clusters compared to each of the labeled clusters. The optimal arrangement of clusters was found by calculating the sum of squared errors for every possible combination of clusters and taking the minimum. Then, the clusters were merged. This method was repeated for every image in the group. Doing this for every color of every species resulted in an output with the number of shades within the iris for each color in each species, as well as an average of each different shade across the data. Throughout this process, images were not resized, in order to allow higher quality images, which have more pixels, to contribute a greater amount to the average.

The final, combined clusters were ranked by how prevalent they were within the eyes, calculated by the number of pixels in each group. The groups for each shade were categorized as

“dark”, “medium”, or “light” according to procedure provided in the Supplementary Methods. The importance of this pipeline is to create a data set that can be compared in a standardized way. The information about which shades are most represented was also collected and saved. This data can be found in the Supplementary Material.

Phylogeny: The phylogeny used for this work was taken from Johnson *et al.* (2006), with the banded linsang (*Prionodon linsang*) as an outgroup (Johnson *et al.* 2006). This is a molecular phylogeny of all eight Felidae lineages with fossil calibrations and usage of Y chromosome data. More recent phylogenies are largely congruent, differing mainly in the placement of the Bay Cat Lineage, due to differences in Y chromosome evolutionary evidence compared to other lines of evidence (Li *et al.* 2016). Both Bay Cat Lineage placements were tested and were found to not produce a significant difference, making the discrepancy irrelevant to this study. Importantly, the Johnson *et al.* tree does not include every extant felid species, nor subspecies, looked at in this study. To remedy this, the missing species and subspecies were added manually according to their placements on the more recent tree from Li *et al.* (2006) or, in the case of subspecies, as a polytomy next to the previously defined species on the tree. Since divergence data was unavailable for some of the species and subspecies, two trees were created. One tree had the species in their proper relationships, but with branch lengths of nearly zero (0.0000001), a severe underestimation of the divergence between groups. This was termed the “short” tree. The other tree also had proper relationships, but with branch lengths equal to the nearest resolved neighboring branch, a severe overestimation of the divergence between groups. This was termed the “long” tree. Completing analyses with both trees and comparing the results bypasses the need for exact branch lengths for the uncertain taxa, provided the results match. A limitation of this method is that it results in uncertainty if the results do not align, which turned out to not be a problem in this case. The “short”

tree with all the eye colors present for each species is shown in Figure 1. The long tree and the short tree created only considering the most common eye colors are presented in Figures S2 and S3.

General color reconstruction: To begin the process of ancestral state reconstruction, the short and long phylogenetic trees were read into R (version 4.2.1) using the package ape (version 5.6-2) (R Core Team 2022; Paradis and Schliep 2019). A table of taxa, and the colors represented for each, was loaded in and scored with 0/1 for absence/presence. The same table with just the most common eye colors was also loaded in.

The command rayDISC() from corHMM (version 2.8) was used for each of the five eye colors independently across the tree (Beaulieu *et al.* 2022). The presence/absence of each eye color was taken on its own, because there would be far too many states to run it as a multistate trait. The accepted state at each node was determined using maximum likelihood methods. All models of trait evolution (equal rates, symmetric rates, and asymmetric rates) were tried, but there were no differences in results. An Akaike information criterion (AIC) analysis done on the results of the fitDiscrete() command from geiger revealed that an asymmetric rates model was best supported, regardless of the lack of difference its use makes in the results (Pennell *et al.* 2014). This process was done for the data of all the observed eye colors, as well as for the data for the most common eye colors.

Exact color reconstruction: After data was collected on the eye colors present for every node on the tree, more specific reconstructions were possible. For each node, a new tree was created for each eye color present at that node. Each new tree included every descendant of that node that shared each eye color with it, except for those where the color was lost and then re-arose independently. For example, an ancestral node that was determined to have hazel/green eyes and

brown eyes present would have one tree with all its continuous, green-eyed descendants and another tree with all its continuous, brown-eyed descendants. A diagram of this method is given in Figure S4.

After the trees were created, the specific colors were reconstructed using maximum likelihood methods with the function fastAnc() from the R package phytools (version 1.2-0) (Revell 2012). This was done independently for the red, green, and blue values for each of the data sets collected for the light, medium, and dark shades. The 95% confidence intervals for the exact reconstructions were extremely wide when using the "short" tree, averaging over -1000 to 1000. Large confidence intervals are a known limitation of continuous trait likelihood reconstructions, but this is particularly egregious given that RGB values can only be from 0-255. However, when the long tree was used, the end results did not change significantly, but the confidence intervals decreased massively, almost always being well within the 0-255 realistic range. This lends considerable support to the reconstructions.

Beyond reconstructing the colors themselves, corHMM's rayDISC() was again used to reconstruct the number of shades within each eye color for each node, using the shade representation data as a discrete, multistate trait. This was also done for the primary and secondary shades within each eye. Put together, these methods allow for a high resolution understanding of the iris color of ancestral felids. For each ancestral felid population, we are able to know: which color eyes were present (out of brown, hazel/green, yellow/beige, gray, and blue), how many different shades they had in their eyes for each color, which shades were more or less common, and approximately what those shades would have been. All of this is present in the Supplementary Material.

Correlation analysis: Apart from reconstructing ancestral states, different correlations were performed in order to investigate the possible evolutionary interactions related to eye color variation. Data on pupil shape was obtained from Banks *et al.* (2015) and data on activity by time of day and primary habitat(s) was obtained from the University of Michigan Animal Diversity Web (Banks *et al.* 2015; Myers *et al.* 2022). Data on zoogeographical regions were based on Johnson *et al.* (2006) and data on coat patterns were based on Werdelin *et al.* (1997). Nose color data (pink or black) and whether or not any black was present in the coat or tail were determined manually from observation of images. These were each converted into a set of binary traits, according to the procedure given in the Supplementary Methods.

This data, along with the presence/absence data for each eye color, was analyzed with a maximum likelihood approach using BayesTraits (version 3.0.5), made accessible in R through the package btw (version 2.0) (Pagel *et al.* 2004; Griffin 2018). This was done by building two models, one where the evolution of two binary traits is independent and one where their evolution is dependent on one another (i.e. where the rate of change in one trait is influenced by the state of the other trait). Then, the models were evaluated using a calculated log Bayes Factor, with a log Bayes Factor over 2 indicating positive evidence for the dependent model. This process was done by comparing the presence of each eye color to all others, as well as the environmental/physical data to the presence of each eye color, the average shade of the RGB values in each eye color, and the average shade of the RGB values in all eye colors overall. This latter average was computed for all taxa by dropping NA values in the averages. To transform the average values into discrete traits, each value was categorized using Jenks natural breaks optimization, performed through the getJenksBreaks() command in the package BAMMtools (version 2.1.10) (Rabosky *et al.* 2014). Finally, tetrachoric correlation coefficients were calculated using the tetrachoric() command in the

package psych (version 2.2.9), to indicate the direction of each association (Revelle 2022). For the shade correlations, a positive association indicates that the trait is associated with lighter shades.

Results

Within the 53 felid taxa considered in the study, gray eyes were found to be present in 39 taxa (74%), brown eyes in 29 taxa (55%), yellow/beige eyes in 24 taxa (45%), hazel/green eyes in 21 taxa (40%), and blue eyes in 7 taxa (13%). These statistics, along with the respective values without counting subspecies and with just counting the most common eye colors are given in Table 1.

Table 1: Eye color analysis count results

Eye Color	In All Felid Taxa	Without Subspecies	Most Common Colors in All Felid Taxa	Most Common Colors Without Subspecies
Gray	39 (74%)	30 (75%)	31 (58%)	22 (55%)
Brown	29 (55%)	27 (68%)	22 (42%)	21 (53%)
Yellow/beige	24 (45%)	16 (40%)	17 (32%)	11 (28%)
Hazel/green	21 (40%)	18 (45%)	11 (21%)	9 (23%)
Blue	7 (13%)	6 (15%)	2 (4%)	2 (5%)

In 10 felid taxa only a single eye color was observed, in 24 taxa two eye colors were observed, in 14 three eye colors were observed, and in 5 four eye colors were observed. When considering just the most common eye colors in the populations of each taxon, 26 felid taxa had only a single eye color, 24 taxa had two eye colors, and 3 had three eye colors. Even with a conservative filtering of the data, there is conclusive evidence of the presence of intraspecific iris color variation among the Felidae.

General color reconstruction: The overall ancestral state reconstruction for all of the colors on the short phylogeny is given in Figure 2, with the presence or absence of each color based on maximum likelihood. The reconstruction for the short phylogeny does not significantly differ from that for the long phylogeny (Fig. S2). The only differences are that, for the long phylogeny, there are fewer ancestors with blue eyes in the *Felis* genus and the common ancestor of *Otocolobus manul* and the genus *Prionailurus* does not have yellow/beige eyes. Most of the colors at each of the nodes have high maximum likelihood support (Fig. S5). However, a notable exception is the ancestor of the Felidae and *Prionodon linsang*. The reconstruction for all five colors is consistently unclear for this node, with presence/absence likelihood values hovering around 0.5.

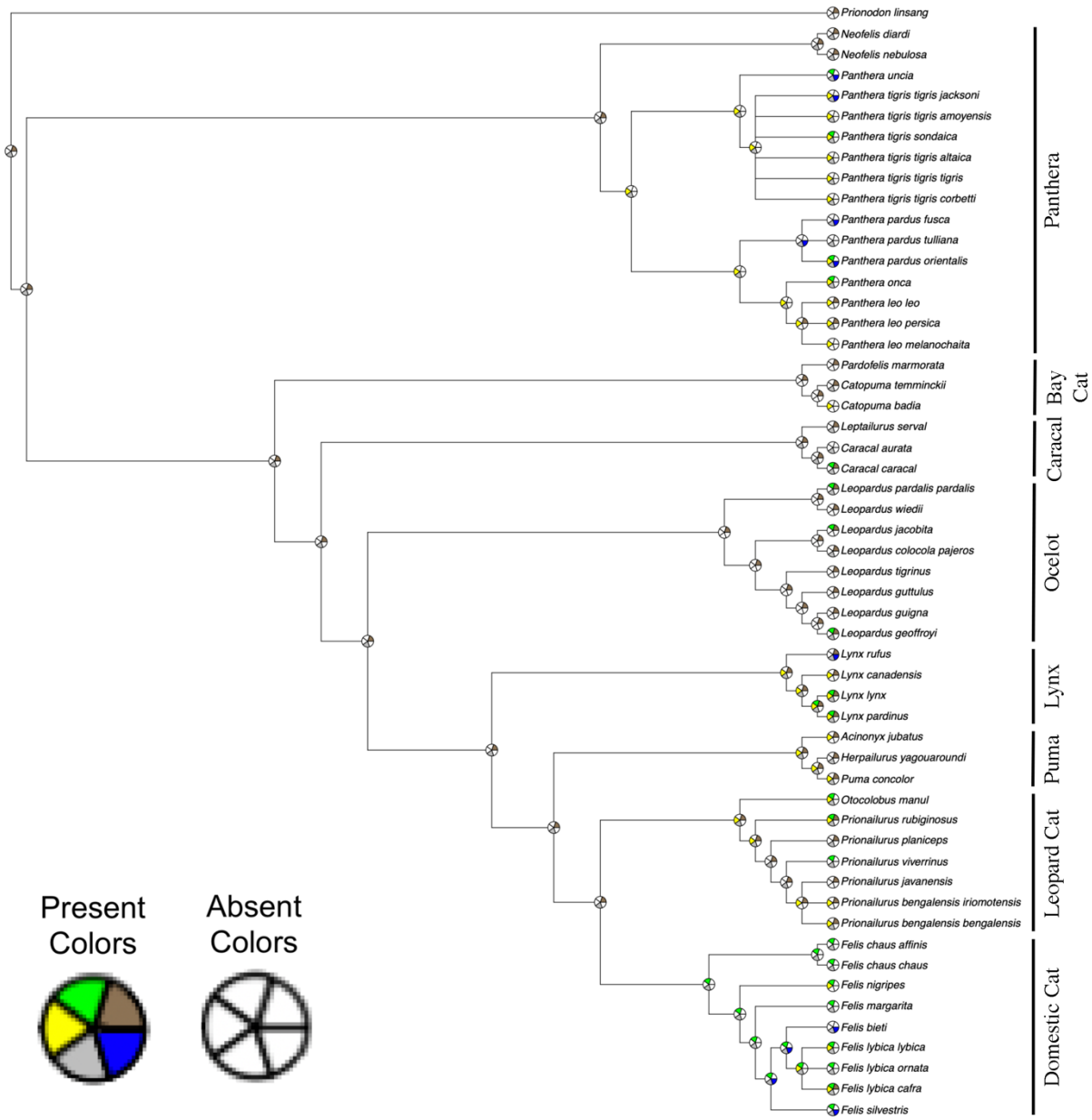

Figure 2: Reconstruction of the ancestral states of all five eye colors. The five-wedge pie charts indicate presence (color) or absence (white) of the various iris colors. Presence information was determined through maximum likelihood support. The tree used is the "short" phylogeny. Exact branch lengths are not plotted and lineage names are given on the right.

The ancestor of the Felidae is reconstructed with high likelihood to have had both brown- and gray-eyed individuals present in its population. This represents two novel adaptations: having multiple eye colors in the same species (intraspecific eye color variation) and having gray eyes in particular. There is good evidence that this is the only major gain of the gray-eyed trait in the Felidae, although a few individual species, such as *Catopuma temminckii* and *Leopardus geoffroyi*, seem to have re-evolved it after a loss. The two largest losses of gray eyes occured when the Bay Cat Lineage and the Ocelot Lineage diverged from the rest of the tree, approximately 9.4 and 8 million years ago, respectively (Johnson *et al.* 2006). Brown eyes are common throughout the tree, but also had two major losses, once when the Domestic Cat Lineage diverged 6.2 million years ago and once after the genus *Panthera* split from *Neofelis* at approximately the same time. There were multiple other species-specific gains and losses, as with gray eyes.

The presence of yellow/beige eyes is predicted to have convergently evolved at least four times in ancestors and multiple more times in individual extant species. The four demonstrated higher than species-level gains of yellow/beige eyes are when the genus *Panthera* split from *Neofelis*, when the Lynx Lineage diverged (7.2 million years ago), when the Puma Lineage diverged (6.7 million years ago), and when the Leopard Cat Lineage diverged (6.2 million years ago). Much like with the other colors, there were a number of cases of loss and even regaining of yellow/beige eyes.

The presence of hazel/green eyes stands apart from the previous three colors because it did not develop early in the evolution and diversification of felids. Instead, it evolved at least twelve individual times, most of the time at the species level alone. The most significant development of hazel/green eyes occurred in the Domestic Cat Lineage when it diverged, only being lost once in that lineage (*Felis bieti*). In fact, that loss is the only observed time hazel/green eyes were ever lost

in the Felidae. The presence of blue eyes has a similar evolutionary distribution to that of hazel/green eyes, albeit much more rare, having evolved independently at least five times. It is only observed in extant species or species-level ancestors, except in the case of the ancestor before *Felis silvestris* (and *Felis catus*, not considered in the phylogeny) split from the rest of the Domestic Cat Lineage. There are only two losses of blue eyes observed (for *Felis lybica* and *Panthera pardus tulliana*).

Exact color reconstruction: The reconstruction of the shades of brown eyes, conducted with reference to the presence/absence reconstruction done above, reveals some large-scale evolutionary trends. It is clear from the eye color of *Prionodon linsang*, as well as its close non-felid relatives that the ancestor of these groups must have had extraordinarily darkly colored brown eyes. This is recapitulated in the reconstruction here (Fig. 3a). The brown eye colors of the common ancestor of the linsangs and the felids are reconstructed as quite dark, albeit not as dark as the irises of *Prionodon linsang*. The brown eyes of the ancestor of the felids has an even lighter coloration, with the light shade being the primary shade in the reconstruction. After this, the proportion of light, medium, and dark shades changes frequently in the tree. In the data, the medium shade is most commonly the primary shade (15 times as opposed to 10 times for the light shade and 5 times for the dark shade). The secondary shade is most commonly the dark shade, but the proportions are close (7 for light, 9 for medium, and 12 for dark). The overall shade of brown eyes (taking into account dark, medium, and light shades for each) also undergoes substantial changes over the tree. In some lineages, such as the Lynx or Ocelot Lineages, the shade returns to a darker state, as it was before felids branched off. In other lineages, such as the Bay Cat Lineage, the shade continued to lighten. It is of interest that, when brown eyes re-evolved in *Felis lybica*

cafra, after their previous loss, it was neither an especially dark nor especially light shade relative to other shades observed on the tree.

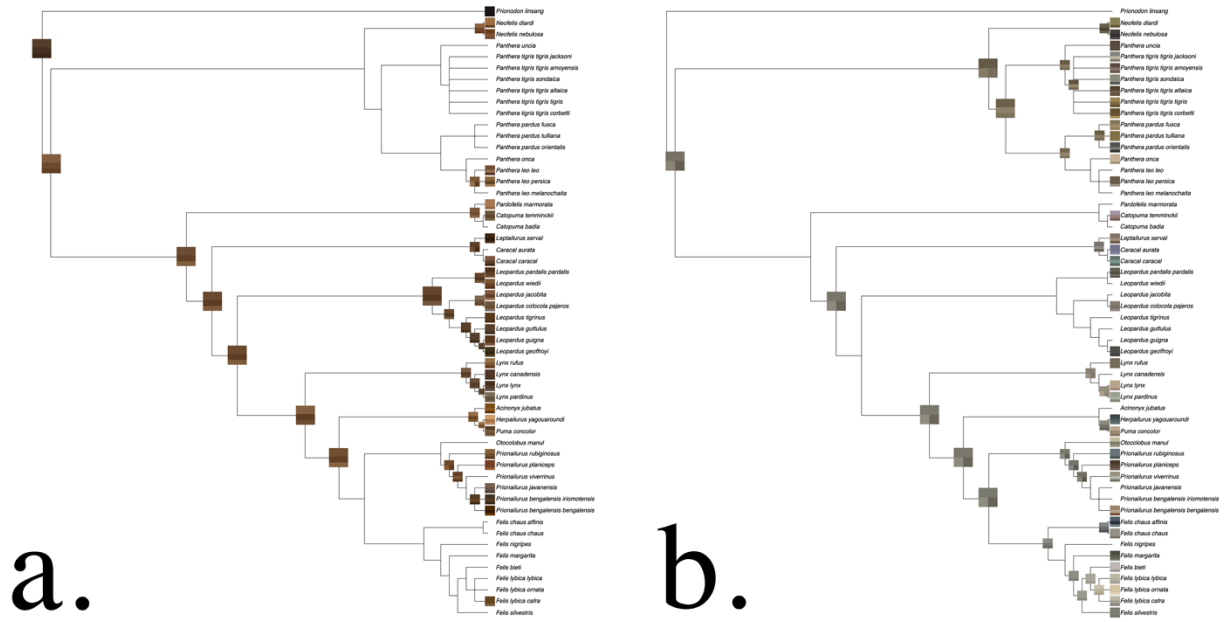

Figure 3: Reconstruction of the ancestral states of the shades of (a) brown and (b) grey eyes. The squares at each node are the exact reconstructed shades. The proportion of a square that a shade takes up indicates how common that shade is in the data. Information was determined through maximum likelihood support. The tree used is the "short" phylogeny. Exact branch lengths are not plotted.

The high level of variation in gray eyes is apparent from viewing the types of gray in the data (Fig. 3b). Unlike brown, where all of the variety was focused within a relatively narrow region, there are gray colors that span a large spectrum, being closer to brown, hazel/green, blue, or yellow/beige for different taxa. The gray that was reconstructed for the ancestor of the Felidae (RGB: 119, 114, 102) is closer to brown-gray than pure gray, a trait that continues as the Felidae diversified. Gray colors have close to equal R, G, and B values, whereas brown colors have much higher R and G values than B. The ancestral gray having a brownish character is evident by the decreased B value, compared to the R and G values. The brown content in gray-eyed animals is

particularly strong in the Panthera Lineage, eventually nearly becoming fully brown for certain taxa. In the Domestic Cat Lineage, the gray color substantially lightened, losing its brown content, particularly for *Felis lybica*. When the genus *Caracal* split from the rest of the Caracal Lineage, its gray changed to have much higher blue and green content (for *Caracal aurata* and *Caracal caracal*, respectively). More blue content in the color of gray eyes is a repeated adaptation, occurring for *Felis chaus affinis*, *Prionailurus rubiginosus*, and *Herpailurus yagouaroundi* as well. As with brown eyes, the medium shade is most commonly the primary shade in gray eyes (15 times), followed by light (11 times), then dark (9 times). This order holds for the most common secondary shades, although it is closer (13, 14, and 10 for light, medium, and dark). Readers interested in the evolution of yellow/beige, hazel/green, and blue eyes are encouraged to look at the Supplementary Results and Figures S6-8.

Correlation analysis: The presence of the five eye colors were correlated against one another, taking into account phylogeny (Fig. 4). The correlations for the "short" phylogeny with all observed eye colors did not significantly differ from the results with only the most commonly observed eye colors (Fig. S9a) nor from the results for the "long" phylogeny (Fig. S9b). A significant positive correlation (log Bayes Factor > 2) was identified every time a color was correlated with itself, a positive baseline check of the quality of the method. The only other significant associations are brown eyes and blue eyes (BF = 7.06) and gray eyes and blue eyes (BF = 4.86), although the association between brown eyes and yellow/beige eyes was close (BF = 1.85). The correlation between brown eyes and blue eyes is negative (corr = -0.63; indeed, only *Lynx rufus* has both brown and blue eyes) and the correlation between gray and blue eyes is positive (corr = 0.97; all blue-eyed taxa also have gray eyes). When the analysis was redone with just the most common eye colors, all significances (or lack thereof) remained the same, except the

brown-yellow/beige correlation, which was found to be significant and negative (BF = 5.62, corr = -0.27).

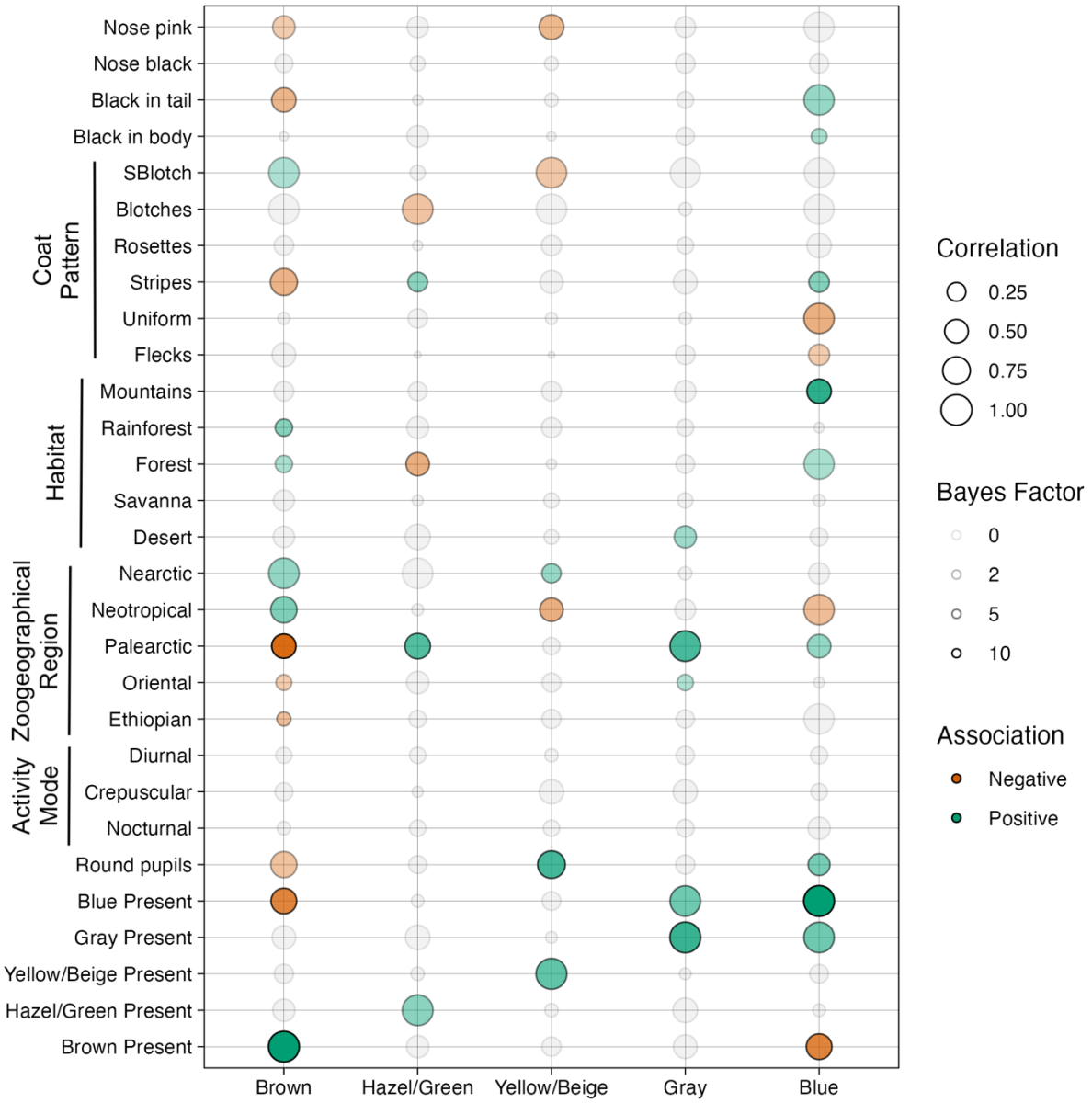

Figure 4: Correlations between the presence of each eye color and various physical and environmental factors. Larger circles correspond to stronger correlations and more opaque circles correspond to more significant correlations. Green circles have a positive correlation, red circles have a negative correlation, and gray circles do not meet the significance threshold (bayes factor = 2). The tree used is the "short" phylogeny.

Overall, most environmental and physical factors considered in the analysis showed at least some significant correlations with various eye colors, indicative of the complexity of eye color evolution. Notably, none of the activity modes were correlated with any eye color, good evidence that this trait is not particularly important for eye color evolution in felids. Another large trend was that, of the significant correlations, gray eyes were only positively associated with other traits. This greatly contrasts with the other eye colors, all of which have closer to a 50/50 distribution of positive and negative associations.

When eye colors were correlated to pupil shape, three significant correlations appeared: brown (BF = 2.72, corr = -0.66), yellow/beige (BF = 6.77, corr = 0.75), and blue (BF = 4.43, corr = 0.40). There is a negative correlation between having brown eyes and round pupils. Only two, closely related taxa evolved round pupils while already having brown eyes (*Acinonyx jubatus* and *Puma concolor*). On the other hand, there is a positive correlation between the presence of yellow/beige or blue eyes and round pupils. It should be noted that one can flip the direction of these correlations to obtain the associations with vertical pupils, given that this is the only other eye option for felids. For other correlation results, see the Supplemental Results.

Discussion

Our results strongly contrast with the assertion given in Negro *et al.* (2017) that "eye colour tends to be a species-specific trait in wild animals, and the exceptions are species in which individuals of the same age group or gender all develop the same eye colour". While this rule may hold for most groups, the Felidae constitute a notable exception, with over 80% of the taxa surveyed in this study having two or more different eye colors in their populations. The images that make up the data set were controlled to be all adults, so this cannot be due to age. Although

the sex of animals in the data set was not able to be determined, sex alone also cannot account for the variation seen, since 35% of taxa had more than two eye colors in their population. Variation in eye colors to this extent has not been formally described, except in humans and domesticated animals, making the felid system an ideal model to investigate the evolution of eye color.

The reconstruction of eye color indicates with high likelihood that the population of the common ancestor of the Felidae had both brown and gray eyed individuals. The presence of brown eyes is not surprising, given that all close relatives of the Felidae have dark brown eyes with no intraspecific variation. However, the presence of gray eyes is likely a family-specific adaptation. Although the program's prediction of whether the ancestor of the Felidae and *Prionodon linsang* had gray eyed individuals is unclear, given that all of the close relatives of the Felidae (e.g. mongooses, hyenas, etc.) have only brown eyes (data not shown), it is highly likely that this uncertain ancestor had only brown eyes as well (Johnson *et al.* 2006).

The gray eye color is likely an intermediate between all of the other eye colors. Eye color is determined by the amounts of the pigments eumelanin and pheomelanin in the iris (Kolb *et al.* 2011). In a simple view, eyes with more eumelanin are brown, eyes with more pheomelanin are yellow, and eyes with lower levels of pheomelanin and eumelanin are blue and green. Gray eyes contain a moderate amount of both pigments, but not enough of either one to reliably be placed in another color group. This is supported by gray eyes in the data having much higher variability than the other four colors. If a population is homogenous for dark brown eyes, such as the relatives of the felids, having a high level of eumelanin and little pheomelanin, it would be difficult to suddenly develop blue eyes, given that blue eyes need a very specific balance between the two pigments that is far from the dark brown state. Even a total loss of pigment, as with albinism, could not

account for this, because a certain amount of pigment is still needed to have the blue color be visible (White and Rabago-Smith 2011).

Under this view, once gray eyes evolved in the felid ancestor, it became far easier to transition between eye colors and evolve new ones, resulting in the great diversification seen in the Felidae. It is out of the scope of this study to answer exactly which genetic changes led to this, but this is a question that should prompt future research. A promising starting point is identifying and comparing orthologous sequence data for genes known to affect melanin production in other species, such as OCA2, HERC2, and MC1R, in as many felid species as possible, to try and pinpoint felid-specific genetic changes that might affect eye colors (White and Rabago-Smith 2011).

Evidence for the evolution of gray eyes being an intermediate form, stemming from a fully brown eyed population, can be found in the shade reconstruction. The ancestral felid gray eyes were not purely gray, but were made up of brownish-gray shades. This is only plausible if there is a gradient from brown to gray with no other colors in between and if the gray eyes were formed from a modified brown eye. Furthermore, when examining the exact shades of gray across the phylogeny, there are places where other colors were lost, coupled with a shift towards that color by gray. For example, in the genus *Panthera*, when brown was lost, there was a concurrent change in the amount of brown in the gray eyed animals. By the present day, the gray eyes in the *Panthera* have almost crossed back into being brown (for example, *Panthera tigris tigris corbetti*). Additionally, there are a number of species for which the content of blue has substantially increased in their gray eyes, such as for *Herpailurus yagouaroundi*, *Felis chaus affinis*, and both species in the genus *Caracal*. However, this is never the case for taxa that already have blue eyes (which all also have gray eyes).

In contrast to *Panthera*, when the Domestic Cat Lineage lost brown eyes, it was coupled with a dramatic lightening of the color of gray eyes and the evolution of hazel/green eyes. In this case, neither of the present colors are close to brown. This represents a second path for the loss of brown eyes: rather than occupying the place of brown eyes in the population by effectively merging brown and gray, the population of the ancestor of the Domestic Cat Lineage shifted the entire eye color scheme. This requires far more changes and it is no wonder that such examples of huge eye color scheme shifts are rare. Through comparisons of this nature, the data collected and analyzed in this study can provide important insights into eye color evolution on both small and large scales. It should also be noted that many of the wild species within the Domestic Cat Lineage can breed with the domestic cat (*Felis catus*) (Oliveira *et al.* 2008; Lyons 2012). Albeit unlikely, disruption of results from this form of hybridization is possible, given that many domestic cats have had artificially selected eye colors.

As for correlations, it is unsurprising that brown and blue eyes, at nearly opposite ends of the pigment spectrum, do not frequently coexist in natural populations and are significantly negatively associated. On the other hand, gray eyes, being an intermediate which is bordering the blue color space, provide an ideal anchor for the rarer blue eyes. The maintenance of blue eyes would be much more likely if blue-eyed individuals mated with blue-eyed or gray-eyed individuals, rather than with brown-eyed individuals. It is worth investigating the only species with both brown and blue eyes, *Lynx rufus*, to see if there is segregation in mating preference along eye color lines. *Lynx rufus* also has gray eyes, which might be a necessary intermediate for the coexistence of blue and brown eyes. Mating preferences provide an intriguing possibility for the evolution of eye color differences, given that human eye colors are likely the product of sexual selection (Frost 2006). Cats are dichromatic and cannot recognize reds and oranges, which fits

well, given that all the eye colors identified in this study are visible to felids and would thus be possible to be sexually selected for (Clark and Clark 2016). However, even if this was the driving factor behind eye color diversification, it still does not explain the emergence of gray eyes, nor the differences between lineages.

Brown eyes are also much less likely to coexist with round pupils. Round pupils are a repeated innovation in felids (the ancestral felid had vertical pupils), but they rarely coincide with brown eyes (Banks *et al.* 2015). There are a few species where they do, but only two had brown eyes before evolving round pupils. It seems probable that an aspect of brown eyes, or a lifestyle that strongly covaries with brown eyes, contrasts with the conditions that are ideal for round pupil evolution. Thus, in most cases, the loss of brown eyes acts as a prerequisite for the evolution of round pupils. Some populations with round pupils re-evolved brown eyes after evolving round pupils (*Panthera leo leo, Panthera leo persica*), but this is rare. The reverse of this trend seems to be true for yellow/beige eyes. Among felids, the evolution of yellow/beige eyes had already happened every time round pupils evolved. The yellow/beige eye color was lost afterwards in the case of *Panthera uncia*, but it was present in the ancestor that evolved round pupils.

The opposing forces of brown and yellow/beige eyes can be seen in the shade correlations, with yellow/beige eyes lightening the overall red and green shades of a species and brown eyes darkening the overall red and green shades. If gray eyes developed by decreasing the amount of eumelanin in the eyes, it could be that a second change increased pheomelanin levels, leading to yellow eyes. Then, with a darker pigmented eye, there might have been less of an evolutionary "need" for eyes with lots of eumalanin. This could explain why many species have either brown or yellow eyes, but not both, particularly in lineages that simultaneously gained yellow and lost brown, such as the genus *Panthera*. The divergent effects of brown and yellow on round pupil

evolution fits in with these two colors being unlikely to develop together, but not being mutually exclusive.

For the activity modes, there were few significant correlations, even with the shade data. This is surprising, given the findings of Passarotto *et al.* (2018), which found that darker colored eyes in owls evolved in response to the switch to a nocturnal lifestyle. This is clearly not the case for felids. The ancestral state for felids is nocturnality, but gray eyes (usually lighter than brown eyes) evolved before any taxa made the switch to diurnality (Myers *et al.* 2022).

The fact that there were many significant correlations by zoogeographical region is fascinating, given how large each region is. This, coupled with the lack of significant correlations found for most habitats and the uniformity of eye colors across most animals around the world, indicates that the physical environment may play less of a direct role on eye color in felids and possibly mammals as a whole. Other traits, such as social system and mode of hunting, are also uninformative, given that the only non-ambush predator felid is the cheetah and the only non-solitary felid is the lion, neither of which show unique eye color characteristics (Banks *et al.* 2015; Myers *et al.* 2022). Thus, the specific adaptive benefit of having different eye colors is left as an open question.

It is known that eye color is at least partly tied to coat color in domestic cats and some such associations do appear in our data, such as having brown eyes being negatively correlated with having a pink nose (Strain 2007). Having a pink nose, an easily measurable partial stand-in for a de-melanated skin color, is unsurprisingly not frequently found in species with brown eyes, which require more melanin. However, for the most part, the color or shade of felid eyes is not related to skin or fur color. This lack of coupling of the two traits, apart from the most melanated cases, likely allowed for the evolution of gray eyes in the felid ancestor.

All of the evidence presented here supports a larger theory of felid eye color evolution. Through random, novel mutation(s) that decreased the levels of iris eumalanin, a subset of the population of the ancestor of the Felidae developed gray eyes. Once this key innovation occurred, the new standing variation led to rapid diversification, creating yellow/beige, hazel/green, and blue eye colors as felid lineages diverged and groups reached new zoogeographical regions. The presence of these colors and the strength of the shades within them varied tremendously through interactions with different environments and physical characteristics. Tradeoffs between the amounts of pigment in the iris created antagonistic relationships between blue and brown eyes, as well as yellow/beige and brown eyes, making their coexistence in various species less likely. The yellow/beige-brown tradeoff, influenced by a potential increase in pheomelanin, possibly affected the development of round pupils. Iris color diversification represents a defining feature of the Felidae family and the data presented here demonstrates the complexity of the trait.

Eyes, and especially eye colors, are a rarely preserved element of animal bodies and have historically been a missed opportunity for evolutionary research. Through this work, the evolution of eye colors in the Felidae is now much clearer and there are many avenues for more studies, particularly regarding the clearly important evolutionary place that gray eyes occupy. This study provides a starting point for future research into eye color evolution in natural populations, a question that has not had any significant investigations until now. Using the methods pioneered in this work, the eye colors of other taxa can be studied in an evolutionary context, without the need for ancient DNA. In addition, the scope of this study could be built upon, adding genetic data to the correlation analysis to try and answer more functional questions. The method for exact color reconstruction in this study could be adapted to any color-based analysis, even beyond the iris. This will allow for high precision color reconstructions that were previously impossible.

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Data Availability

The code and all the raw images in the data set used for this article are freely available on GitHub at https://github.com/jtabin/Felid-Eyes.

Supplementary Material

Data, supplementary methods and results, and supplementary figures are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.s4mw6m9b0.

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