Evolutionary Insights Into Felidae Iris Color Through Ancestral State Reconstruction

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Summary

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 quantitatively reconstructed. The ancestral felid population was predicted 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 among 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. This diversity of human eye colors, ranging from brown to green to blue, has been attributed to sexual selection (Frost 2006). 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).Yet, such intraspecific eye color variation has been described as rare amongst animals, apart from artificially selected domesticated animals (Negro *et al.* 2017). Moreover, 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 has been the belief 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" (Negro *et al.* 2017).

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 – although the closest relatives to the felids, such as linsangs, hyenas, and genets (Johnson *et al.* 2006), all have brown eyes and little inter- or intraspecific variation, 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 and help interrogate how and why the current variants 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.

Results

To assess the range of eye colors present in the Felidae, we leveraged high-quality public image databases and sampled individuals from all non-domesticated felid species, as well as four related outgroups (see Methods for details). Which eye colors are present for each taxa was determined impartially using color identification software (Methods). Within the 52 felid taxa considered in the study, gray eyes were found to be present in 38 taxa (73%), brown eyes in 28 taxa (54%), yellow/beige eyes in 23 taxa (44%), hazel/green eyes in 21 taxa (40%), and blue eyes in 7 taxa (13%). These statistics are given in Table 1, along with the results when subspecies are not considered and when just the most common eye colors (eye colors present in >80% of each taxon's individuals in the surveyed databases; see Methods) are considered.

In 10 felid taxa only a single eye color was observed, in 24 taxa two eye colors were observed, in 13 three eye colors were observed, and in 5 four eye colors were observed. All the eye colors present for each species are displayed in Figure 1. When considering just the most common eye colors in the populations of each taxon, 25 felid taxa had only a single eye color, 24 taxa had two eye colors, and 3 had three eye colors. Even with this conservative filtering of the data, there is conclusive evidence of the presence of intraspecific iris color variation among the Felidae.

General color reconstruction: Eye color was reconstructed by subsetting a published ultrametric Carnivora supertree (Nyakatura and Bininda-Emonds 2012; see Methods for details). This "main phylogeny" is nevertheless missing nine extant felid taxa, which were added manually (Methods) to generate an expanded "full phylogeny". The overall ancestral state reconstruction for all of the colors on the main phylogeny is given in Figure 2, with a color considered present if there was greater than 60% support as determined by maximum likelihood methods. This reconstruction does not substantially differ from the reconstruction for the full phylogeny, with additional species and subspecies added (Fig. S2). The only differences are that, for the full phylogeny, there are fewer blue-eyed ancestors on the tree and there is more confidence that the common ancestor of the Panthera lineage had yellow/beige eyes. Most of the presence/absence information for the colors have high maximum likelihood support across the tree (Fig. S4). However, a notable exception is hazel/green. Under the main tree, taking into account all observed eye colors, the confidence in the presence of hazel/green is around 50% for every node, likely due to the wide and seemingly unpatterned distribution of hazel/green eyes across taxa. This lack of confidence in the presence of hazel/green eyes is ameliorated when either using only the most common eye colors or the full phylogeny. In both cases, the analysis

predicts the presence of hazel/green eyes in the ancestor of the Domestic Cat lineage with high confidence. Apart from these few areas, all three analyses are congruent. The eye color of the ancestor of the Felidae and *Prionodon linsang*, or any of the deeper nodes on the tree, are not worth considering here, given that ancestral state reconstruction becomes more uncertain the farther back one goes. The reconstruction for all five colors are consistently unclear for these nodes.

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 changes: 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 subsequently lost it. Brown eyes are also common throughout the tree, but had two major losses, once when the Domestic Cat Lineage diverged 6.2 million years ago and once after the Panthera Lineage diverged 10.8 million years ago (Johnson *et al.* 2006). However, there is uncertainty and discordance between the three analyses about whether the Panthera Lineage ancestor had brown eyes, so this latter brown eye loss may have occurred when the genus *Panthera* split from *Neofelis* approximately 6.5 million years ago. As with gray eyes, there were multiple other species-specific losses, as well as a regaining of brown eyes by *Panthera leo*.

The presence of yellow/beige eyes is predicted to have convergently evolved at least twice in ancestors and multiple more times in individual extant species. The two demonstrated higher than species-level gains of yellow/beige eyes are when the genus *Panthera* split from *Neofelis*, and when the Lynx, Leopard Cat, Domestic, and Puma Lineages diverged from the rest

of the tree (8 million years ago) (Johnson *et al.* 2006). Much like 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 does not seem to have developed early in the evolution and diversification of felids. Instead, it appears to have evolved at least twelve individual times, most of the time at the species level alone. According to the most common color and full phylogeny analyses, 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, possibly having evolved independently at least four times, according to the main analysis: in *Lynx rufus*, in the ancestor of the *Panthera* genus, in *Panthera pardus*, after a prior loss, and in the ancestor of *Felis silvestris* and *Felis bieti* (also the ancestor of *Felis catus*, not considered in the phylogeny). It should be noted that the most common color and full phylogeny analyses both lead to an alternative prediction that the blue eye color was not present in any of the ancestors and arose in individual tips independently.

Quantitative color reconstruction: Beyond assigning eye color to one of the aforementioned five broad color groupings, RGB values from each image were processed using a dimensionality reduction algorithm and examined using a cluster analysis (see Methods for details). This resulted in a quantitative and finely detailed output of the average number of shades for each eye color in each taxon, as well as what the colors of those shades are. The reconstruction of the shades of brown eyes, conducted with reference to the presence/absence reconstruction done above, reveals some large-scale evolutionary trends. The eye color of the

outgroups (*Vulpes zerda, Crocuta crocuta*, *Genetta genetta*, and *Prionodon linsang*), as well as their close non-felid relatives that were not analyzed in this study, suggests that the ancestor of these groups likely had extraordinarily dark colored brown eyes. Our study does not have the requisite power to confirm the presence of a brown-eyed ancestor this deep in the tree, but it would be surprising if similar dark brown eyes convergently evolved across closely related and ecologically distinct species. Assuming that the ancestor of the outgroups and the Felidae had brown eyes, the predicted darkness is recapitulated in the reconstruction (Fig. 3a). The brown eye colors of all the outgroup ancestors are reconstructed as quite dark, albeit not as dark as the irises of *Prionodon linsang*. The brown eyes of the ancestor of the felids are predicted to have had a lighter coloration, but still with the dark shade being the primary shade in the eye. After this, the proportion of light, medium, and dark shades changes frequently in the tree. In the data, the three shades have about an equal distribution of being the primary shade (8 for light, 9 for medium, and 8 for dark). The secondary shade is most commonly the dark shade (3 for light, 6 for medium, and 16 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.

A 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, 112, 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 and sometimes taking on a slightly higher green content, particularly for *Felis margarita*. 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 trait, occurring for *Prionailurus rubiginosus* and *Herpailurus yagouaroundi* as well. Here, the medium shade is most commonly the primary shade in gray eyes (17 times), followed by dark (5 times), then light (4 times). This order holds for the most common secondary shades, although it is closer (6, 11, and 9 for light, medium, and dark). Similar analyses of the evolution of yellow/beige, hazel/green, and blue eyes are shown in the Supplementary Results, as well as Figures S5-7.

Correlation analysis: The presence of the five eye colors were correlated against one another, taking the phylogeny into account (Fig. 4). The correlations for the main phylogeny with all observed eye colors sometimes differ from the results with only the most commonly observed eye colors (Fig. S8a) or for the full phylogeny (Fig. S8b), but often there is agreement. For all three analyses, 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 main analysis demonstrated a significant negative correlation between the presence of brown eyes and the presence of both hazel/green (BF = 7.30, corr = -0.71) and blue (BF = 6.27, corr = -0.75; indeed, only *Lynx rufus* has both brown and blue eyes). In contrast, the presence of gray

eyes is significantly positively correlated with the presence of both hazel/green (BF = 7.04, corr = 0.63) and blue (BF = 6.19, corr = 0.98; all blue-eyed taxa also have gray eyes). These correlations for gray, as well as the negative brown-blue correlation, were found for other two analysis types as well. However, the analysis with just the most common eye colors indicated additional negative correlations between brown eyes and both yellow/beige (BF = 9.76, corr = -0.78) and gray eyes (BF = 6.88, corr = -0.67) .

Correlation analysis was also carried out on eye color relative to a variety of other physical traits (e.g. pupil shape, coat color), behavioral traits (e.g. diurnal versus nocturnal activity), and habitat characteristics. 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, except for crepuscularity with gray eyes—good evidence that this trait is not particularly important for eye color evolution in felids. Additionally, of the significant correlations, gray eyes were almost always 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, one significant correlation appeared: the presence of yellow/beige eyes is strongly positively correlated with round pupils (BF = 6.90, corr = 0.80). This correlation was found in the most common eye color analysis as well. Additionally, in that analysis, there was a significant negative correlation between having brown eyes and round pupils (BF = 2.92, corr = -0.70). This makes sense as only two taxa evolved round pupils while already having brown eyes (the closely related *Acinonyx jubatus* and *Puma concolor*). 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.

There were some correlations between zoogeographical regions and various eye colors that cannot be otherwise explained by phylogeny, particularly on the ends of the color spectrum: brown eyes are positively associated with the nearctic region (BF = 3.39, corr = 0.97), gray eyes are positively associated with the palearctic region (BF = 8.24, corr = 0.99), and blue eyes are negatively associated with the neotropical region (BF = 3.73, corr = -0.98). On the other hand, there were few correlations with habitat. For more on these results, including the shade correlations given in Figure S9, see the Supplementary Results.

Discussion

Our results 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 stage of maturation. Although the sex of animals in the data set could not 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 common ancestor population for 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 characteristic. Although our analysis has left it unclear whether the ancestor of the Felidae and *Prionodon linsang* included gray eyed individuals, given that all of the close relatives of the Felidae (e.g. genets, hyenas, etc.) have only brown eyes, it is highly likely that this uncertain ancestor had exclusively 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 or 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).

Further 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 quantitative 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*). 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*, *Prionailurus rubiginosus*, and both species in the genus *Caracal*. However, this is never the case for taxa that already have blue eyes, all of which 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 likely 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 surely 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 from this form of hybridization is possible, given that many domestic cats have had artificially selected eye colors.

The correlation results were also revealing. 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, but can distinguish other colors (Clark and Clark 2016). This range of color sensitivity 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. 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, when the predominant eye colors in taxa are taken into account. Round pupils are a repeated innovation in felids (the ancestral felid had vertical pupils; Banks *et al.* 2015), but they rarely coincide with brown eyes. 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 (e.g. *Panthera leo*), 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 green and blue 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.

There were few significant correlations for activity modes, even with the shade data taken into account. 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 a number of 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 long-distance (as opposed to short-range 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 independent 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 quantitative 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.

Limitations of the Study

While ancestral state reconstruction can provide statistical insight into the past, it remains a prediction. This study does not conclusively prove that the ancestor of the Felidae had gray eyes; it strongly predicts that this was the case, given modern data. To be more sure about the eye colors of felid ancestors would likely require a firm knowledge of which genes influence modern felid eye color and how their pathways interact, as well as extensive ancient DNA sampling of extinct felids. This would allow the predictions made by the models used here to be empirically tested.

Additionally, despite online databases providing essential support for this work, using such databases limits the amount of data that can be collected on possible confounders. For instance, the age, sex, and provenance of the animals whose photos were used for this study could not be reliably determined. While these factors should not have affected the overall conclusions of this study, data was not able to be collected about how eye color varies on these biologically-relevant axes.

Lastly, the sampling done for this study is only sufficient to make robust predictions about the Felidae. For a researcher interested in species beyond this family, more data would have to be collected and analyzed using the methods described in this article.

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Author Contributions

Conceptualization, J.A.T.; data collection and preparation, J.A.T. and K.A.C.; data analysis, J.A.T.; writing – original draft, J.A.T.; writing – review & editing, J.A.T. and K.A.C.; supervision, J.A.T.

Declaration of Interests

The authors declare no competing interests.

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 12 identifiable subspecies and four outgroups (banded linsang, *Prionodon linsang*; spotted hyena, *Crocuta crocuta*; common genet, *Genetta genetta*; and fennec fox, *Vulpes zerda*), 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 56 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 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 279 cropped, standardized, 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 with all recorded eye colors, as well as using only the most common eye colors, thereby assuring that rare eye colors did not skew the results.

Shade measurements within each color group: For each species, the images were sorted into their color groups. 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), a method selected for its

preservation of local structure, important for potential fine shade differences (McInnes *et al.* 2018). The UMAP projection 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. This was done to ensure any blurring from lower quality images did not obscure the true shade variety in each eye.

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 the 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 subset from the Carnivora supertree from Nyakatura and Bininda-Emonds (2012). This ultrametric phylogeny takes into account 188 literature and gene trees and includes members of all eight Felidae lineages. More recent phylogenies are largely congruent, differing mainly in the placement of the Bay Cat Lineage and the Pallas’s cat (*Otocolobus manul*), partly due to differences in Y chromosome evolutionary evidence compared to other lines of evidence (Li *et al.* 2016). Alternate placements were tested and were found to not produce a significant difference, making these discrepancies irrelevant to this study.

This Carnivora supertree tree is missing 9 of the extant felid groups for which data was collected. Thus, a second tree (termed the “full” tree) was created with the missing species being added manually according to their placements on a Felidae specific tree from Johnson *et al.* (2006) and/or the more recent tree from Li *et al.* (2016). The subspecies added were defined according to the most recent identification based on Kitchener *et al*. (2017) and Liu *et al.* (2018). Subspecies were added as a polytomy next to the previously defined species on the tree. Since divergence data was unavailable for some of the species and subspecies, the additions were made with branch lengths equal to the nearest resolved neighboring branch, a severe overestimation of the divergence between groups.

It is important to note that this method of manually adding taxa to a tree is flawed without proper sequence data and certainly should not be relied upon for ancestral state predictions or to

make broad claims, as there is no guarantee that any addition reflects true divergence. However, this tree was created purely to try and provide some insight into local areas of the tree at the species level (e.g. what was the eye color of the ancestral tiger?). Even still, these predictions must be understood as far more uncertain than analyses with the original supertree with more limited taxa. The main tree with all the eye colors present for each species is shown in Figure 1. The full tree and the main tree created only considering the most common eye colors are presented in Figure S2.

General color reconstruction: To begin the process of ancestral state reconstruction, the 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). Although the presence/absence of each eye color were analyzed on their own, the colors are likely not fully independent. Therefore, they were also analyzed together as a polymorphic trait using stochastic mapping through fitpolyMk() from the R package phytools (version 1.2-0) (Revell 2012). Since there were far too many states (2^5-1), including high parameter complexity, for adequate interpretation as a polymorphic character and the two analyses generally aligned (data not shown), the independent model was used for the rest of the analysis. A color was said to be present at any given node (Figure 2) if the marginal maximum likelihood ancestral state reconstruction for that color was 60% or greater. The optimal model of trait evolution was determined using an Akaike information criterion (AIC) analysis done on the results of the fitDiscrete() command from

geiger comparing equal/symmetric and asymmetric rates (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 and for the full phylogeny, with the AIC output and weights given in Supplementary Table 2.

Quantitative 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 of these subset trees 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 S3. This method was done to most accurately reconstruct along plausible evolutionary pathways. If one wants to predict the eye shade of a specific color for a specific node, one should omit taxa that either have lost that eye color (since their present condition cannot communicate any relevant information about the shade of that color for their ancestor), as well as taxa that have lost that eye color and then regained it (since it is unknown whether their present condition is at all related to the shade of that color for their ancestor).

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. Since RGB values can only be from 0-255, it was heartening that the 95% confidence intervals for the quantitative reconstructions were almost always well within the realistic range, lending considerable support to the

reconstructions. Large confidence intervals are a known limitation of continuous trait likelihood reconstructions, so one should not understand the reconstructions to always communicate the exact eye shades of the felid ancestors, but they are useful in comparison to one another to illuminate larger trends.

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. Given the stochasticity of these models, the model comparisons were done 100 times and the calculated log Bayes Factors were averaged, ensuring robust and reproducible results. 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.

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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.

Figure Legends

Figure 1: Phylogenetic tree of the Felidae (and outgroups), modified from Nyakatura and Bininda-Emonds (2012), 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.

Figure 2: Reconstruction of the ancestral states of all five eye colors for the Felidae. The five-wedge pie charts indicate presence (color) or absence (white) of the various iris colors. Colors were considered present if there was >60% support as determined by maximum likelihood methods (probabilities plotted in Figure S5). Exact branch lengths are not plotted and lineage names are given on the right.

Figure 3: Reconstruction of the ancestral states of the shades of (a) brown and (b) gray eyes. The squares at each node are the quantitative reconstructed shades. The proportion of a

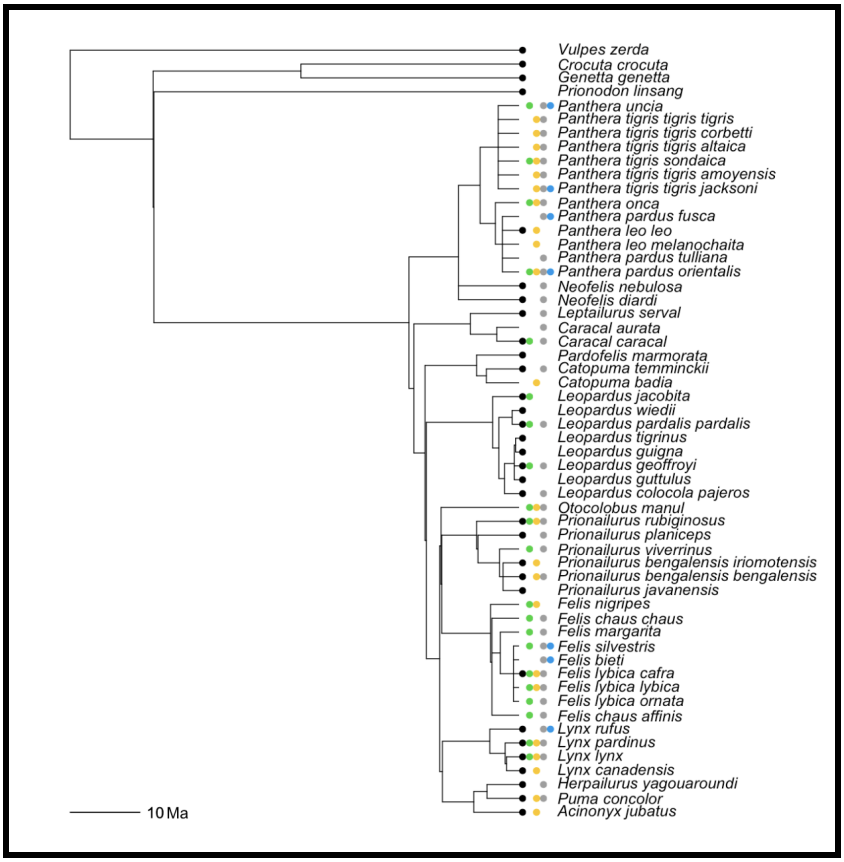
square that a shade takes up indicates how common that shade is in the data. Exact branch lengths are not plotted. The square highlighted in red is the ancestor of the Felidae.

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).

Tables

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	38 (73%)	31 (72%)	31 (60%)	23 (53%)
Brown	28 (54%)	28 (65%)	22 (42%)	22 (51%)
Yellow/beige	23 (44%)	17 (40%)	16 (31%)	10 (23%)
Hazel/green	21 (40%)	20 (47%)	11 (21%)	10 (23%)
Blue	7 (13%)	6 (14%)	2 (4%)	2 (5%)



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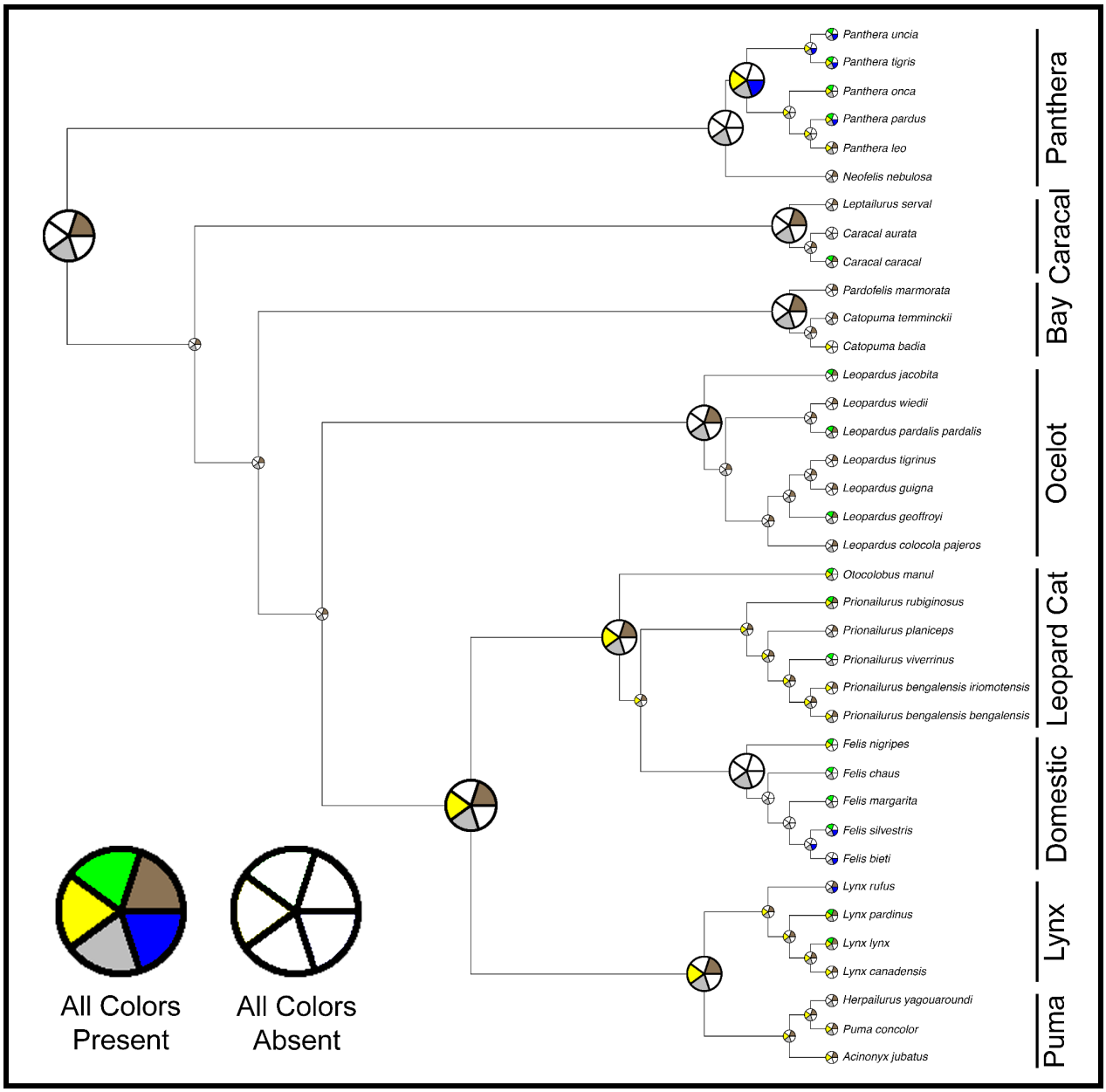

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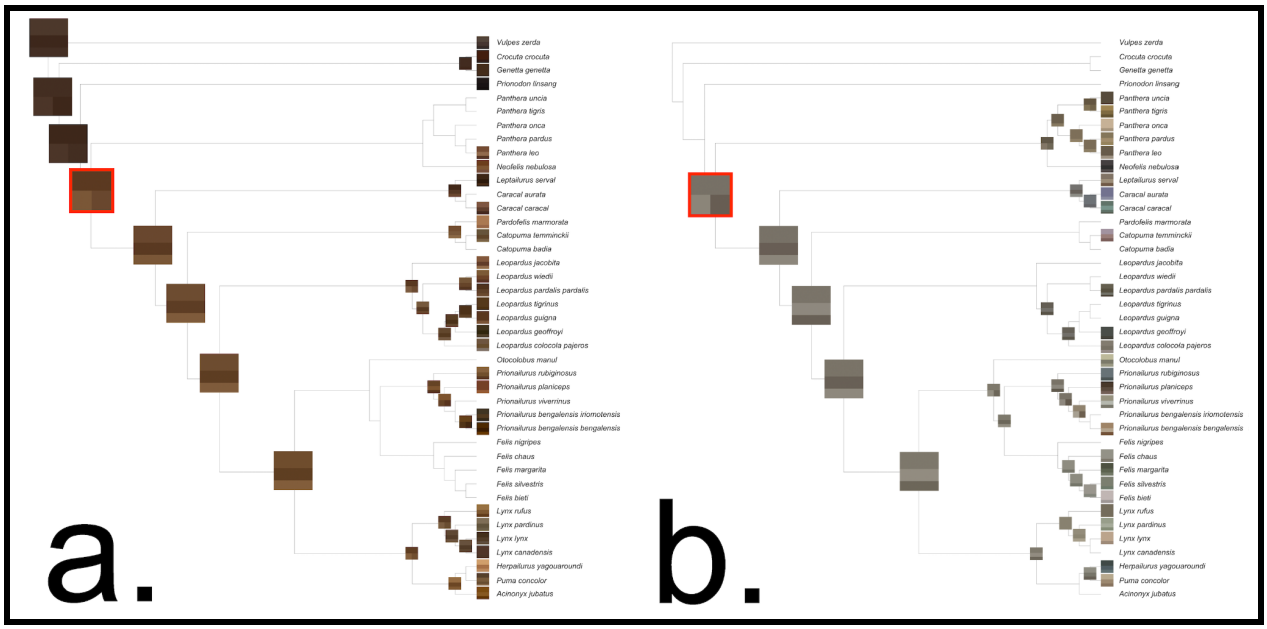
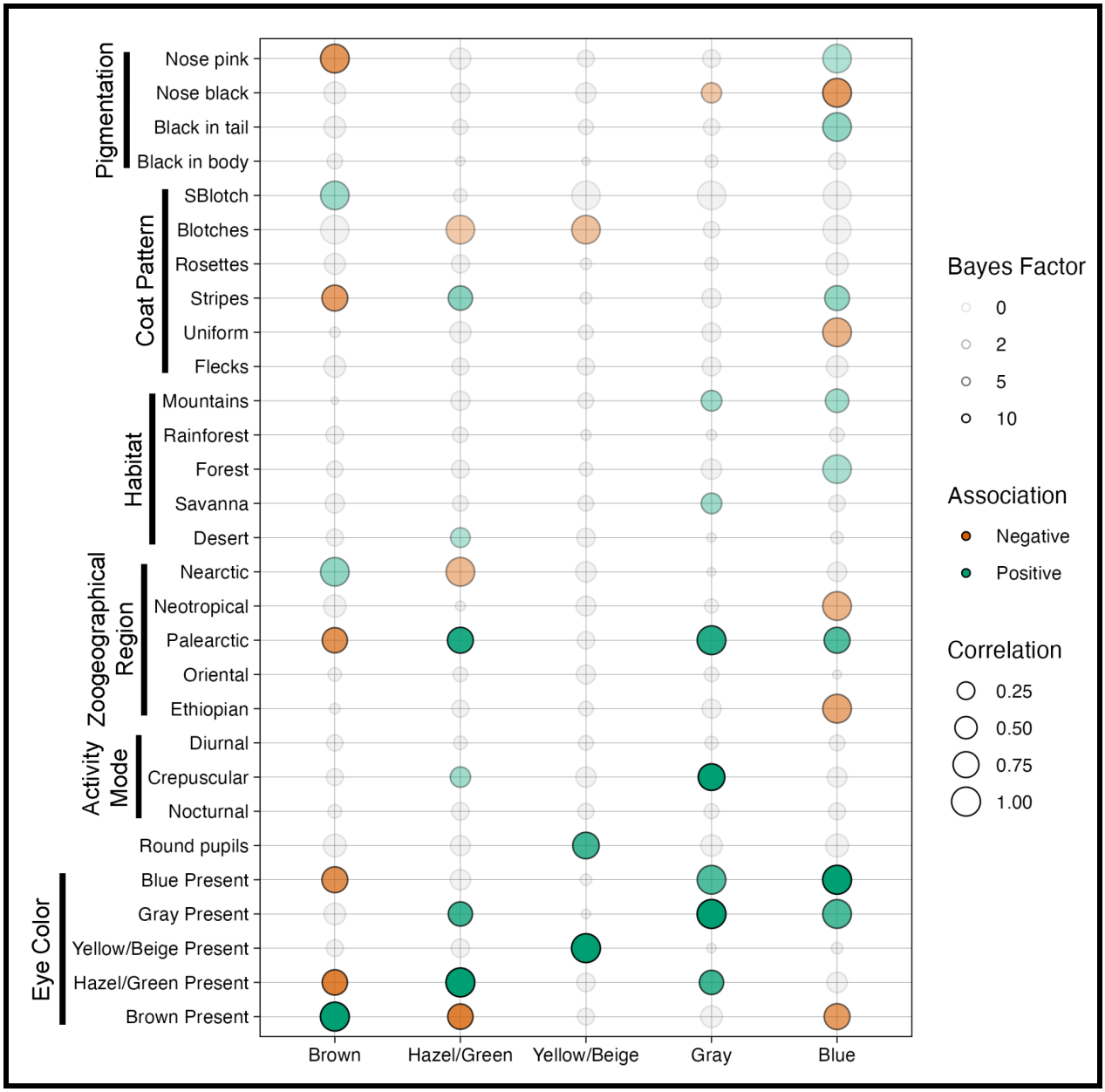


Figure 3: Reconstruction of the ancestral states of the shades of (a) brown and (b) gray eyes. The squares at each node are the quantitative reconstructed shades. The proportion of a square that a shade takes up indicates how common that shade is in the data. Exact branch lengths are not plotted. The square highlighted in red is the ancestor of the Felidae.



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