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The evolution of peafowl and other taxa with ocelli (eyespots): a phylogenomic approach

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The most striking feature of peafowl (*Pavo*) is the males' elaborate train, which exhibits ocelli (ornamental eyespots) that are under sexual selection. Two additional genera within the Phasianidae (*Polyplectron* and *Argusianus*) exhibit ocelli, but the appearance and location of these ornamental eyespots exhibit substantial variation among these genera, raising the question of whether ocelli are homologous. Within *Polyplectron*, ocelli are ancestral, suggesting ocelli may have evolved even earlier, prior to the divergence among genera. However, it remains unclear whether *Pavo*, *Polyplectron* and *Argusianus* form a monophyletic clade in which ocelli evolved once. We estimated the phylogeny of the ocellated species using sequences from 1966 ultraconserved elements (UCEs) and three mitochondrial regions. The three ocellated genera did form a strongly supported clade, but each ocellated genus was sister to at least one genus without ocelli. Indeed, *Polyplectron* and *Galloperdix*, a genus not previously suggested to be related to any ocellated taxon, were sister genera. The close relationship between taxa with and without ocelli suggests multiple gains or losses. Independent gains, possibly reflecting a pre-existing bias for eye-like structures among females and/or the existence of a simple mutational pathway for the origin of ocelli, appears to be the most likely explanation.

1. Introduction

> As no ornaments are more beautiful than the ocelli on the feather of various birds . . . they deserve to be especially noticed. [1, p. 132]

The wide diversity of colour and ornamental traits in birds has long been of interest to both scientists and non-scientists. Indeed, the ornamental traits of birds were instrumental to Darwin's development of the theory of sexual selection [1], where he devoted four chapters to the subject. The striking train of male peafowl, comprising elongated tail coverts decorated with ocelli (ornamental eyespots), had a special place within this discussion of avian sexual selection. Assuming gradual evolutionary change, Darwin recognized that there must have been species of birds with successive steps between 'the magnificent ocelli of [peacocks] and the simpler ocelli or mere coloured spots of other birds' ([1], p. 137).

To gather evidence of their gradual evolution, Darwin studied the striking ocelli present on male peafowls (*Pavo* sp.) in addition to similar structures found among species of two other genera in the same order (Galliformes) and family (Phasianidae): *Polyplectron* (peacock-pheasants) and *Argusianus* (argus pheasants). He immediately noted significant variation in the size, number and coloration of ocelli among these genera in addition to observing differences in the distribution of ocelli among taxa—*Argusianus* have ocelli located on the wing feathers and *Polyplectron* have ocelli distributed among different feather types (including tail, tail coverts, flight feathers, wing coverts

and the mantle). Darwin also noted that females either lack ocelli (*Pavo* and *Argusianus*) or have smaller, less complex and/or less iridescent ocelli (*Polyplectron*) than males. It has since become clear that the ocelli of *Pavo* species are involved in sexual selection [2–4] and that male display of ocelli towards females during mating [5] also suggests a role for ocelli in sexual selection among *Argusianus* and *Polyplectron*. Indeed, males in all of these taxa prominently display ocelli towards females during mating displays [5], often with careful orientation to maximize impact of the ocelli [6]. Darwin postulated that the ocelli of these taxa were homologous (using modern terminology), suggesting a single origin and then subsequent modification and elaboration via sexual selection.

Darwin's hypothesis predicts that modern, phylogenetic analyses should strongly support a close relationship among galliforms having ocelli. However, the inferred relationships among these taxa remain controversial. Although there is strong support for a peafowl clade (*Pavo* and *Afropavo*, the Congo peafowl) [7–10] and an argus clade (*Argusianus* and *Rheinardia*, the crested argus) [5], the relationships between these two clades and *Polyplectron* have varied (reviewed in [9]). A few studies unite the peafowl, argus and peacock-pheasants into an 'ocellated clade' [5,10], though with little support; a larger number of studies unite the peafowl and argus clades to the exclusion of the peacock-pheasants [9,11–13], whereas others do not unite any of these three clades [14]. The position of *Polyplectron* is especially variable among studies [9], suggesting that large amounts of data may be necessary to resolve this question.

To further complicate matters, both the peafowl and argus clades contain one genus with ocelli (*Pavo* and *Argusianus*) and one genus lacking ocelli (*Afropavo* and *Rheinardia*), suggesting either a loss in *Afropavo* and *Rheinardia* or independent gains of ocelli in *Pavo* and *Argusianus* [7]. Ocelli also occur in the earliest diverging members of the genus *Polyplectron* [5] but two derived species (*Polyplectron inopinatum* and *Polyplectron chalcurum*) exhibit reduced or no ocelli, suggesting loss of ocelli in this genus. Additionally, recent studies using a limited amount of mitochondrial data suggest *Haematortyx* (crimson-headed partridge, which lacks ocelli) might be closely related to *Polyplectron* [14,15]. If correct, this relationship provides another example of a highly elaborated, dimorphic group closely related to a species with little elaboration or dimorphism, further emphasizing the lability of secondary sexual traits in galliforms [14,16]. The difficulties in resolving phylogenetic relationships among the ocellated galliforms combined with the potential loss of ocelli within genera have made it unclear whether ocelli are homologous (with subsequent loss in some taxa) or whether ocelli arose independently in each ocellated genus.

Here, we ask whether the ocellated galliform taxa form a clade and determine the closest relatives of the enigmatic genus *Polyplectron*. To accomplish this, we used a supermatrix analysis including all galliform species with available sequence data (233 species) to select 15 target species that represent: (i) taxa with ocelli, (ii) the potential sister taxa of any ocellated genera and (iii) several appropriate outgroup species (electronic supplementary material, table S1). We inferred evolutionary relationships among these 15 target species using high-throughput sequencing of ultraconserved elements (UCEs) and their flanking DNA. Finally, we reconstructed the ancestral condition of ocelli to examine whether ocelli are likely to have evolved once or multiple times.

2. Material and methods

(a) Supermatrix analyses to identify target taxa

To identify all possible sister taxa of the ocellated taxa, we consulted Wang *et al.* [9], who reviewed previous galliform phylogenies, and we used available data to construct a mitochondrial supermatrix of *CYB*, *ND2* and *12S* rRNA sequences from GenBank for Galliformes (electronic supplementary material, table S2). We supplemented those data with mitochondrial sequences collected by different methods, including high-throughput sequencing [17] and PCR amplification from toe pads (see [18] for methods). The final matrix included 233 species, corresponding to 83% of Galliformes and 91% of Phasianidae (the family that includes the ocellated taxa).

Using this mitochondrial supermatrix, we performed unpartitioned and partitioned (by gene and codon position) maximum-likelihood (ML) analyses using RAxML v. 7.3.0 [19]. We analysed the mitochondrial DNA using the best model (GTR + I + Γ), identified using the Akaike information criterion (AIC) in MODEL-TEST v. 3.06 [20] and assessed support using 1000 bootstrap replicates. We also used Bayesian inference (BI) to assess support, conducting analyses in MRBAYES v. 3.1.2 [21] on the CIPRES Science Gateway [22] using the GTR + I + Γ model. We ran the analyses for 10^7 generations using the standard settings (two runs, each with four heated chains) and we sampled every 1000 generations. The first 25% of the sampled trees were discarded as burn-in.

(b) Ultraconserved element data collection

We selected 15 species for UCE enrichment using a combination of literature searches and the trees inferred above. These taxa included representatives of the three ocellated genera, putative sister taxa and multiple outgroups. We collected UCE data using a modification of the Faircloth *et al.* [23] approach. Briefly, we prepared Nextera sequencing libraries using the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). We pooled libraries into groups of eight taxa, enriched each library pool for 5060 UCE loci (targeted by 5472 probes), amplified the enriched pools using limited-cycle PCR (18 cycles), quantified the resulting pools using qPCR (Kapa Biosystems) and sequenced the enriched libraries using a single Illumina HiSeq 2000 lane (PE75 reads generated at the UC Irvine Genomics High-Throughput Facility). We conducted de novo assembly using Velvet [24], matched the contigs to defined UCE loci using PHYLUCE v. 1.1 [23], and added UCE sequences from the *Gallus gallus* and *Meleagris gallopavo* genome assemblies [25,26]. We excluded loci having missing data for any taxon and aligned the sequences using MAFFT [27]. We examined alignments by eye in GENEIOUS PRO (v. 5.6.6) and removed likely assembly or alignment errors at the 5′ or 3′ ends prior to subsequent analyses.

(c) Phylogenomic analyses of ultraconserved element data

The 15-taxon dataset comprised 1966 UCE loci after excluding loci with missing data in any taxon, producing a completely sampled matrix. The average locus length was 409 bp (range = 344–746 bp) and the concatenated alignment contained 803 901 sites (17 559 were parsimony informative). We conducted unpartitioned and partitioned ML analyses and BI using the GTR + I + Γ model (the best-fitting based on the AIC [20]). Importance values of the base composition parameters for individual UCEs were much higher than those for other parameters (electronic supplementary material, figure S1) and GC content was the axis for which parameter estimates exhibited the greatest variation (electronic supplementary material, figure S2). Therefore, we partitioned the UCE data based upon GC content, using

five bins (30–38%, 38–46%, 46–54%, 54–62% and 62–70% GC) containing varying numbers of loci.

We implemented the SMRT-ML [28] method to estimate the species tree using the UCE data. SMRT-ML is a method for analysing concatenated data that is consistent when gene tree topologies differ due to incomplete lineage sorting under the multispecies coalescent. This allowed us to use of all of the data and avoid including poorly resolved gene trees as is done in a standard coalescent-based analysis. We chose this approach because the coalescent-based analysis in a previous UCE study [29] had limited resolution, possibly reflecting, at least in part, the inclusion of poorly resolved gene trees. To implement SMRT-ML, we wrote a perl script that identified ML trees (using GTR + I + Γ without partitioning in RAxML v. 7.2.8 [19]) for each possible rooted triple and then combined quartettes using the modified MinCut method [30]. We estimated support using 100 bootstrap replicates generated prior to running the SMRT-ML script.

To provide a taxon-rich dataset with the power to reconstruct the phylogeny of the ocellated taxa, we combined the 233-taxon mitochondrial matrix with the 15-taxon UCE dataset. This dataset included all eight of *Polyplectron* and *Pavo*, two (of three) species of *Galloperdix*, as well as *Argusianus*, *Rheinardia* and *Haematortyx*. The optimal tree was estimated using RAxML and the GTR + I + Γ model with 10 randomized starting trees. Species represented only by mitochondrial data had longer branch lengths than those represented by both mitochondrial and UCE data. Therefore, the branch lengths for the combined UCE + mitochondrial topology were re-estimated using only mitochondrial data.

Presence and absence of ocelli was scored as a binary trait. Although the ocelli in *P. inopinatum* are reduced (simpler than in some of the other species), they were scored as present. To reconstruct ancestral states using ML, we used MESQUITE 2.72 [29] and the STOCHCHAR package [29] to determine whether a two-rate model (gains ≠ losses; AsymmMk) was significantly better than a one-rate model (gains = losses) with a likelihood ratio test. After identifying the best model (the AsymmMk model), we reconstructed the evolution of ocelli using the STOCHCHAR package.

3. Results

(a) Identification of genera related to ocellated taxa

The ML and BI phylogenies from the 233-taxon mitochondrial dataset strongly supported both a peafowl (*Pavo–Afropavo*) clade and an argus (*Argusianus–Rheinardia*) clade (figure 1 and the electronic supplementary material, S3), as expected. We also found strong support for an expanded peacock-pheasant clade comprising *Polyplectron*, *Galloperdix* and *Haematortyx*. Similar to previous studies [5,7,16], the mitochondrial data did not resolve relationships among the ocellated clades with high support (figure 1 and the electronic supplementary material, S3). Based on these results, we sampled UCE loci from *Haematortyx* and *Galloperdix* along with the ocellated taxa. To these taxa, we added additional phasianid taxa ($n = 7$) that were sufficient to test whether the topology for the ocellated taxa corresponded to that in any recent study (reviewed in [9]) as well as a non-phasianid outgroup.

(b) Resolving relationships among genera using ultraconserved elements

In contrast to previous studies and mitochondrial data (figure 1), which have been unable to resolve relationships among the ocellated taxa, we found a strongly supported
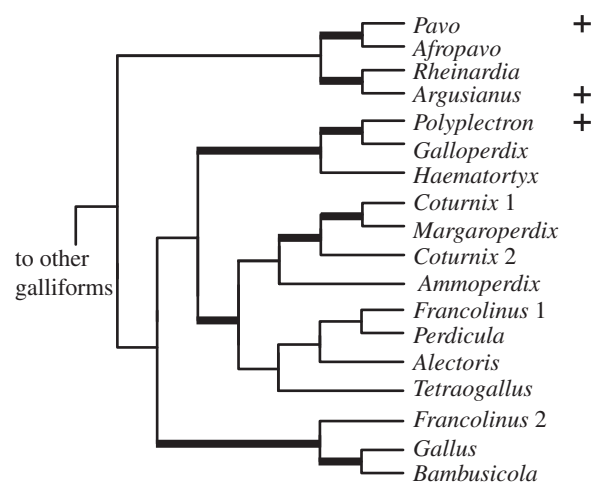

Figure 1. Tree based upon ML analysis of the mitochondrial *CYB*, *ND2* and *12S* genes. Only the clade containing the taxa of interest is shown; the complete phylogeny is shown in the electronic supplementary material, figure S2. Nodes that are supported by more than 70% bootstrap values in ML analysis and also have BI posterior probabilities of 0.85 or greater are indicated by thicker lines. Plus symbols (+) indicate presence of ocelli.

clade uniting the ocellated taxa in both the ML and BI analyses of the UCE dataset (figure 2). As we found with the mitochondrial data, analyses of the UCEs suggested that *Polyplectron*, *Haematortyx* and *Galloperdix* form an expanded peacock-pheasant clade, united with the peafowl and argus clades. Many previous studies, including other studies with limited numbers of nuclear loci, have failed to support monophyly of the clade that contains *Pavo*, *Argusianus* and *Polyplectron* [9]. This raised the question of whether our support for this clade reflects the change in taxon sampling, which breaks up the long branch to *Polyplectron*. However, the optimal tree united the three clades even when we excluded *Haematortyx* and *Galloperdix* (electronic supplementary material, figure S4), suggesting the topological differences among previous studies reflect the use of insufficient amounts of data. Finally, we note that the SMRT-ML tree also provided strong support for the clade comprising the ocellated taxa (figure 2), strongly suggesting that this clade is present in the species tree.

Multiple hypotheses can explain the distribution of ocelli (figure 3). However, ancestral state reconstruction using likelihood was equivocal regarding the presence or absence of ocelli in the common ancestor of *Pavo*, *Argusianus* and *Polyplectron* (electronic supplementary material, figure S5). In spite of this, our phylogenetic analyses do provide a strongly supported hypothesis of relationships among these taxa that can be used to examine alternative models of evolution of ocelli (as discussed below).

4. Discussion

(a) Relationships among ocellated taxa

Our results provide strong support for a clade that includes all three genera of galliforms (*Pavo*, *Argusianus* and *Polyplectron*) that exhibit ocelli. However, we found that the clade also includes additional genera that lack ocelli. In addition to the expected sister relationships between *Pavo–Afropavo* and *Argusianus–Rheinardia*, we also found that *Polyplectron* is
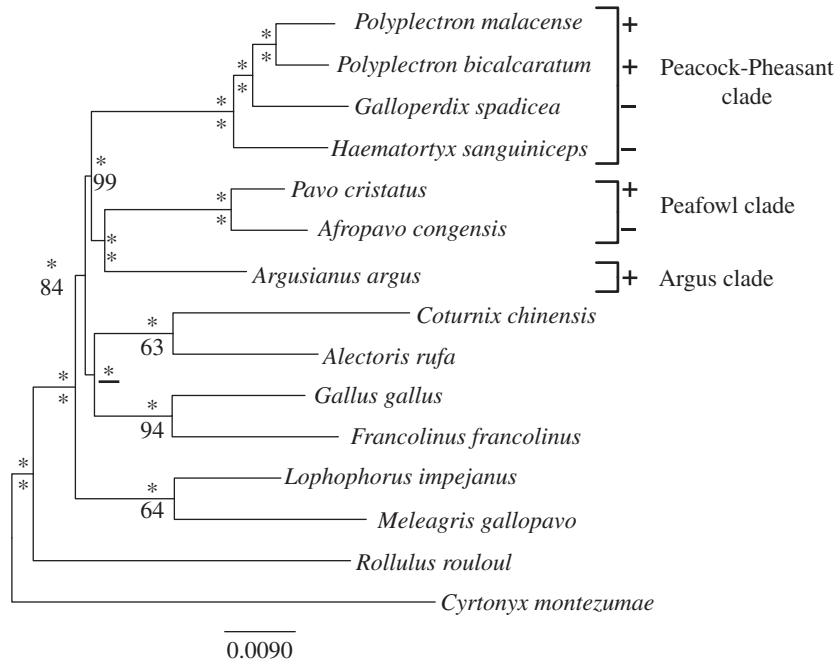
Figure 2. ML phylogeny estimated from UCEs. An asterisk (*) represents 100% ML bootstrap support. Values above the nodes are those from the partitioned RAxML analysis, whereas values below the nodes are from the SMRT-ML analysis (one rearrangement, with low support, differed in this analysis and is indicated with a minus symbol (−)). Partitioned ML, unpartitioned ML analyses and BI produced the same topology and all nodes had 100% bootstrap or posterior probabilities of 1.0. For the clade of interest, plus symbols (+) indicate presence of ocelli and minus symbols (−) indicate the absence of ocelli.
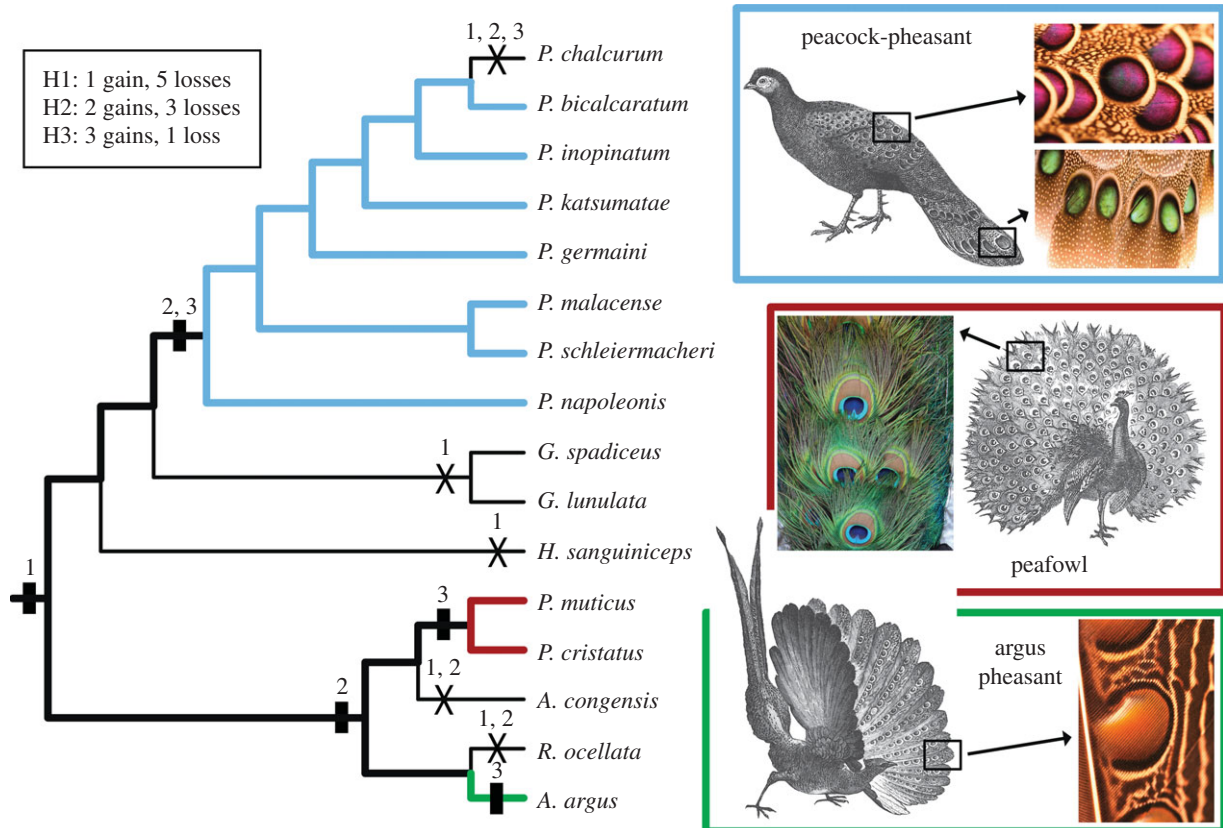

Figure 3. ML cladogram estimated from the combined mitochondrial and UCE data showing the focal taxa; thin branches lead to taxa that lack ocelli. Gains of ocelli are marked with a bar and losses are marked with cross symbols (X). Numbers above each gain or loss mark indicate which hypothesis or hypotheses (noted in upper left) the mark refers to. Images of the ocellated taxa, including close-ups of the ocelli, are shown to the right. For the peacock-pheasants, where ocelli occur on multiple feather types, both the single ocelli (found on most feather types) and the double ocelli (found on tail feathers) are shown; although the colours differ between the photographs they were actually of the same individual and emphasize the different appearances of iridescence depending upon the angle of the light. The photographs of *Argusianus argus* and *Polyplectron bicalcaratum* ocelli were provided by Hannah Owens; the *Pavo cristatus* image was taken from a set of public domain images uploaded to http://pixabay.com by user Efraimstochter. (Online version in colour.)

closely related to two non-ocellated genera: *Galloperdix* and *Haematortyx*. Two recent studies have suggested *Haematortyx* might be related to *Polyplectron* based upon limited mitochondrial data [14,15], but the close relationship of *Galloperdix* with these two genera was unexpected based upon the literature. Although *Galloperdix* and *Haematortyx* do exhibit sexual dimorphism, both genera have typically been considered partridges. By contrast, the other genera in this clade, including those without ocelli, are pheasant-like taxa that have specialized feathers and ornamentation.

(b) Evolution of ocelli

The simplest modern interpretation of Darwin's [1] hypothesis, that the ocelli of *Pavo*, *Argusianus* and *Polyplectron* are homologous, would suggest the existence of a clade comprising the ocellated taxa and excluding other galliforms. This simple interpretation was falsified by the support for close relationships between ocellated (*Pavo* and *Argusianus*) and non-ocellated (*Afropavo* and *Rheinartia*) taxa [5,7]. We further falsified this simple hypothesis when we found strong support for placing *Galloperdix* and *Haematortyx* as successive sister groups of *Polyplectron*. However, Darwin's [1] hypothesis does not preclude the inclusion of other taxa in this clade. Previous evidence for the loss of ocelli in *P. chalcurum* [5] raises the possibility that ocelli are homologous (i.e. there was a single gain) but that the single gain may have been followed by multiple losses.

With the potential for both loss and gain of ocelli, there are three different hypotheses that are consistent with our phylogeny (figure 3). Hypothesis 1 assumes a single gain, consistent with Darwin's [1] hypothesis, but then requires five subsequent losses (six total evolutionary steps). Hypothesis 2 is an alternative that requires two independent gains and at least three losses, resulting in five steps. However, the most parsimonious model of ocellus evolution (hypothesis 3) requires only four steps, three independent gains in each of the three ocellated genera and a single loss within the genus *Polyplectron*. Darwin's [1] hypothesis (our hypothesis 1) might be favoured if losses occur much more frequently than gains. The relatively short terminal branch length for *P. chalcurum* [5] indicates that loss of ocelli can occur relatively rapidly. By contrast, the rate of gain is less clear. If hypothesis 1 is correct, ocelli would have to be gained along the very short branch uniting the ocellated clade (figure 2). By contrast, the hypothesis of multiple origins would allow gain along the relatively long branches leading to each ocellated genus (figure 2), providing few constraints upon the rate of gain for these characters.

Given that a simple likelihood reconstruction does not completely address the evolution of ocelli, it is clearly desirable to incorporate other types of biological information to understand the evolution of ocelli. There are differences in the appearance and distribution of ocelli among feather types in *Pavo*, *Argusianus* and *Polyplectron*. Indeed, Darwin [1] stated that the existence of single ocelli in *Pavo* but paired ocelli in *Polyplectron* (figure 3; though we note that paired ocelli only occur on the tail feathers) made him question the hypothesis that the traits were related. Although homologous traits typically vary in appearance among species, the location of the ocelli also varies among the taxa of interest in this study (tail coverts in *Pavo*, greatly elongated secondaries in *Argusianus*, and broadly distributed on the

body in *Polyplectron*; figure 3). These differences in distribution could be suggestive of multiple origins. Indeed, given the differences among taxa in the distribution of ocelli, the hypothesis of a single origin would require either (i) an ancestor with ocelli on multiple feather types (similar to most *Polyplecton* species) combined with loss on most feather types in *Pavo* and *Argusianus* or (ii) an ancestor with ocelli on a single feather type combined with shifts among feather types (i.e. the development of ocelli on a novel feather type combined with the loss of expression on the original feather type). Thus, even if Darwin's [1] hypothesis of homology were correct (hypothesis 1), the evolution of galliform ocelli must be more complex (and therefore even less parsimonious) than a single origin and five losses.

There are multiple explanations for the loss of ocelli. Kimball *et al.* [5] presented strong evidence for the loss of ocelli within the genus *Polyplectron*, noting that both the species that has lost ocelli and a second species with simplified ocelli have montane distributions, unlike the lowland distribution of the ocellated *Polyplectron* species. Reduced male ornamentation with increased altitude has been observed in other birds [31], possibly reflecting a reduction in the strength of sexual selection and/or the increased cost of trait production due to resource limitation. However, the other members of the larger clade that lack ocelli (*Afropavo*, *Rheinardia*, *Galloperdix* and *Haematortyx*) inhabit lowlands. Therefore, even if a single origin of ocelli is correct, one cannot invoke altitudinal shifts to explain loss of ocelli in these taxa. Nonetheless, there may be additional, as yet unknown, factors that could have an impact upon the strength of natural and/or sexual selection leading to the loss of ocelli in these species.

The most parsimonious hypothesis, which invokes multiple independent gains of ocelli (and a single loss within *Polyplectron*, see figure 3, hypothesis 3), is potentially of greater interest. Modified feathers and featherless regions on the head of many phasianid species highlight the eyes during displays. In addition, female preference for traits that emphasize the eye have been identified (e.g. [32–34]), suggesting there may be a general female preference for eye-like structures [35]. Thus, female phasianids may have a pre-existing sensory bias [36] for eye-like structures. Any genetic mutations in males that lead to eye-like structures or exaggerate the prominence of those structures might be favoured by sexual selection. If there are relatively few mutational events necessary to evolve ocelli (potentially expanding simple spots to a complex ocelli), then it is possible that this pathway has occurred repeatedly on different feather types among these taxa.

5. Conclusion

Phylogenomic methods, such as sequence capture [23], make it possible to obtain large amounts of data to infer phylogenetic relationships with a high degree of confidence. Although previous galliform phylogenies, including multilocus studies, have been equivocal regarding the relationships among ocellated taxa, the UCE data provide strong support for a clade that includes the three ocellated genera. The data also strongly support the inclusion of taxa without ocelli within the clade. Despite the well-resolved phylogeny, ancestral state reconstruction remained equivocal. Given

valid explanations for both multiple losses as well as multiple gains, Darwin's [1] hypothesis cannot be fully addressed with phylogeny alone. However, considering other types of information suggests that multiple gains may be likely. Improvements in our understanding of feather development [37] may allow an examination of the developmental pathways underlying the presence of ocelli (versus spots and other feather features) to further our understanding of the evolution of ocelli. We believe that this is likely to be a fruitful next step now that the phylogeny of this group has been established with confidence.

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